The effect of different soil yeasts on the growth and physiology of lupin and wheat

by Leandra Moller

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DECLARATION

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This dissertation includes one original paper published in a peer-reviewed journal and one unpublished publication (of which the information is being used in a provisional patent application). The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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Declaration by the candidate:

With regard to chapter 2 and 3 the nature and scope of my contribution were as follows:

Nature of contribution	**Extend of contribution (%)**
Planning and execution of experimental work, data analyses and interpretation, and manuscript writing	80 %

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The undersigned hereby confirms that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to chapters 2 and 3
2. No other authors contributed to chapters 2 and 3 besides those specified above, and
3. Potential conflicts of interested have been revealed to all interested parties and that the necessary arrangement have been made to use the material in chapters 2 and 3.

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SUMMARY

Leguminous plants are often used in break-crop and crop rotation systems to fix atmospheric di-nitrogen (N_2), thereby reducing the need for nitrogen (N) fertiliser input during cereal production. In these systems, wheat (*Triticum aestivum* L.) yield increases following blue lupin (*Lupinus angustifolius* L.) cultivation. Yields can also be increased by plant growth promoting microorganisms such as yeasts. These plant growth promoting yeasts (PGPY) enhance seed germination, produce plant growth promoting (PGP) factors, and partake in beneficial tripartite and quadripartite symbioses with their host plant and its root symbionts. Despite the importance of blue lupin in agriculture, it was unclear how tripartite and quadripartite interactions involving PGPY affect this legume's biological N_2 fixation (BNF) and growth. In addition, the effect of a single PGPY on germination and growth of plants used in break-crop or crop rotation systems, such as blue lupin and wheat, was unknown. To address these aspects, the impact of a rhizosphere yeast and mycorrhizal fungi on growth and nutrition of nodulated blue lupin was firstly evaluated. The ability of this rhizosphere yeast to affect a break-crop or crop rotation system was subsequently assessed by studying the yeast's influence on the germination and developmental physiology of blue lupin and wheat.

Papiliotrema laurentii (syn. *Cryptococcus laurentii*) was isolated from the rhizosphere of blue lupin. Nodulated blue lupin seedlings, treated with either *P. laurentii* CAB 91 (PL), or mycorrhizal fungi (MF), or with both symbionts (PLMF), were then cultivated in a glasshouse, after which the plants' biomass parameters, symbiotic colonisation, and mineral nutrition were analysed. The PGP traits of PL were subsequently evaluated by comparing them to that of two other rhizosphere yeasts, *i.e. Hannaella zeae* CAB 1119 (HZ) and *Saitozyma podzolica* CAB 1199 (SP). The yeasts were used to coat seeds of blue lupin and wheat, where after their influence on the germination of these seeds was assessed under controlled conditions. The cold test was used to evaluate the influence of the three yeast strains on the vigour of both plants. To determine the effect of HZ, PL, and SP on growth and photosynthesis of nodulated blue lupin and wheat, plants were cultivated under glass house conditions.

It was found that the MF treatment had no effect on blue lupin seedlings under glass house conditions. Improved growth of PL treated seedlings was underpinned by

increased BNF efficiency, while greater nodulation and efficient growth on N resources supported the increased biomass of PLMF treated plants. Subsequent trials with the three rhizosphere yeasts mentioned above, indicated that of these three yeasts only PL can be used to coat the seeds of both blue lupin and wheat, since this yeast increased the germination of blue lupin and the vigour of wheat. Additionally, under glass house conditions PL promoted higher relative growth rates during the early developmental stages of both plants. The latter coincided with enhanced photosynthetic metabolism and water relations. These findings indicate that PL may potentially serve as an efficient bio-fertiliser of blue lupin and wheat to benefit break-crop and crop rotation systems.

OPSOMMING

Peulplante word dikwels gebruik in breekgewas- en wisselboustelsels om atmosferiese stikstof (N_2) te bind, wat die behoefte aan stikstof (N) kunsmis-insette tydens graanproduksie verminder. In hierdie stelsels word die opbrengs van koring (*Triticum aestivum* L.) verhoog na die verbouing van blou lupiene (*Lupinus angustifolius* L.). Opbrengste kan ook verhoog word deur plant-groei-bevorderende mikroörganismes, soos giste. Hierdie plant-groei-bevorderende giste (PGBG) verbeter saadontkieming, produseer plant-groei-bevorderende (PGB) faktore en is betrokke by voordelige drie- en vierledige simbiosis met hul gasheerplant en sy wortelsimbionte. Ten spyte van die belangrikheid van blou lupien in die landbou bedryf, was dit onduidelik hoe drie- en vierledige interaksies waarby PGBG betrokke is, die biologiese N_2-fiksering (BNF) en groei van die peulplant beïnvloed. Daarbenewens was die effek van 'n enkele PGBG op ontkieming en groei van plante wat in breekgewas- of wisselboustelsels gebruik word, soos blou lupiene en koring, onbekend. Om hierdie aspekte aan te spreek, is die impak van 'n rhisosfeergis en mikorisas op die groei en voeding van blou lupiene in die teenwoordigheid van wortelknoppie-bakterieë in hierdie studie geëvalueer. Vervolgens is die vermoë van hierdie rhisosfeergis om 'n breekgewas- of wisselboustelsel te beïnvloed, beoordeel deur die gis se invloed op die ontkieming en ontwikkelingsfisiologie van blou lupiene en koring te bestudeer.

Papiliotrema laurentii (sinoniem *Cryptococcus laurentii*) is vanuit die rhisosfeer van blou lupiene geïsoleer. Blou lupiene-plantjies, gekoloniseer deur wortelknoppie bakterieë, is behandel met óf *P. laurentii* CAB 91 (PL), óf mikorisas (MF), óf met beide simbionte (PLMF). Die plantjies is daarna in 'n glashuis gekweek, waarna die plante se biomassa-veranderlikes, simbiotiese kolonisasie en minerale voeding geëvalueer is. Die PGB-eienskappe van PL is vervolgens geëvalueer deur die eienskappe te vergelyk met dié van twee ander rhisosfeergiste, nl. *Hannaella zeae* CAB 1119 (HZ) en *Saitozyma podzolica* CAB 1199 (SP). Die giste is as saadbedekking van blou lupiene en koring gebruik, waarna hul invloed op die ontkieming van hierdie sade onder beheerde toestande beoordeel is. Die koue toets is gebruik om die invloed van die drie gisstamme op die groeikragtigheid van albei plantsoorte te evalueer. Die plante is onder glashuistoestande gekweek om die effek van HZ, PL en SP op die groei en fotosintese van blou lupiene en koring te bepaal.

Daar is gevind dat die MF-behandeling geen invloed op blou lupiene-plantjies onder glashuis-toestande gehad het nie. Verbeterde groei van PL-behandelde saailinge is ondersteun deur verhoogde BNF-doeltreffendheid, terwyl meer wortelknoppies en doeltreffende groei op N-bronne die verhoogde biomassa van PLMF-behandelde plante ondersteun het. Daaropvolgende proewe met die drie bogenoemde rhisosfeergiste het aangedui dat slegs PL as 'n saadbedekking vir beide blou lupiene en koring gebruik kan word, aangesien hierdie gis die ontkieming van blou lupiene en die groeikragtigheid van koring verbeter het. Verder, onder glashuistoestande het PL albei plante se relatiewe groeitempo tydens die vroeë ontwikkelingsfases verhoog. Laasgenoemde het gepaard gegaan met verbeterde fotosintetiese metabolisme en water verbruik. Hierdie bevindinge dui daarop dat PL moontlik aangewend kan word as 'n doeltreffende bio-kunsmis vir blou lupiene en koring, om breekgewas- en wisselboustelsels te verbeter.

Dedicated to my parents,

for all the sacrifices you made to further my education

"Come forth into the light of things,
let Nature be your teacher."
–William Wordsworth

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MOTIVATION

Triticum aestivum L. (wheat) is an important food source and the second most cultivated grain crop in the world (FAO 2017). Due to the demand for wheat-based foods, farmers often over-apply nitrogen (N) fertilisers to soil to ensure high wheat yields (Kong et al. 2017); a practice that may incur multiple undesirable effects on wheat growth (Yang et al. 2000, 2004; Bundy and Andraski 2004; Hawkesford 2014; Kong et al. 2017), which eventually results in reduced yields. This paradox, together with the negative impact of N fertilisers on the environment (Vitousek et al. 1997; Adesemoye and Kloepper 2009), has shifted the focus towards using sustainable agricultural practices to increase wheat yields. One such a strategy is the use of break-crop and crop rotation systems, where grains, such as wheat, is cultivated in a systematic manner with unrelated plant species to enhance soil fertility, while reducing disease (Kirkegaard et al. 2008; Angus et al. 2015). Leguminous plants are often used in these systems, since they are able to fix di-nitrogen (N_2) via biological N fixation (BNF), thereby reducing N inputs during wheat cultivation (O'Donovan et al. 2014; Preissel et al. 2015; St. Luce et al. 2015).

A legume that has been successfully used in these systems, is *Lupinus angustifolius* L. (blue lupin), due to it being capable of growing in nutrient depleted soils (Lambers et al. 2013). This is owed to its ability to obtain N via BNF (Kim and Rees 1994; Zahran 1999) and phosphorous (P) through carboxylate exudation from the roots (Egle et al. 2003; Lambers et al. 2013). This plant is not only important from an agricultural perspective, however, since its seeds are regarded to be a health food for humans (Stephany et al. 2016; Hane et al. 2017). Considering that the world population is estimated to reach 9.60 billion people by 2050 (FAO and ITPS 2015), and that both blue lupin and wheat is used for human consumption, it is clear that production of both of these plants will have to be increased in future. It is expected that increased crop production will mostly stem from heightened intensification (Bruinsma 2009), *i.e.* higher cropping intensities and yields.

The need to increase crop yields in a sustainable manner has been the driving force behind the interest in using plant growth promoting microorganisms, such as plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi, as bio-fertilisers (Timmusk et al. 2017). This is due to the knowledge that PGPR can increase

germination, growth, nutrition and photosynthesis of plants (Parray et al. 2016), while mycorrhizal fungi are known to improve growth and nutrition of their host plants (Balestrini and Lumini 2017). Yet, soil yeasts may also influence the growth (Amprayn et al. 2012; Deng et al. 2012; Wang et al. 2013; Mukherjee and Sen 2014; Fu et al. 2016; Liu et al. 2016; Silambarasan and Vangnai 2017), nutrition (Cloete et al. 2009, 2010a; Azcon et al. 2010; Nakayan et al. 2013; Morsy et al. 2014; Kang et al. 2015) and photosynthesis (Cloete et al. 2010b) of different plants, due to their possession of plant growth promoting (PGP) traits. Some of these plant growth promoting yeasts (PGPY) are known to improve seed germination (Gaballah and Gomaa 2004; Shalaby and El-Nady 2008; Akhtyamova and Sattarova 2013; Nakayan et al. 2013; New et al. 2013; Matić et al. 2014; Ramos-Garza et al. 2016), which may result in greater yields. Yet, *in vitro* germination test results do not always represent germination performance in the field (ISTA 2014; Marcos Filho 2015; Finch-Savage and Bassel 2016), which is more accurately represented by seed vigour (ISTA 2014). However, to date no study has focussed on the impact of PGPY on seed vigour or on the germination of blue lupin and wheat. Although it was demonstrated that PGPY may participate in quadripartite interactions with legumes, rhizobia and mycorrhizal fungi (Singh et al. 1991; Medina et al. 2004; Azcon et al. 2010), it is unclear whether blue lupin is able to form such a symbiosis and if this interaction would impact BNF, growth and nutrition of the plant. Despite evidence that PGPY may increase wheat growth (Perondi et al. 1996; Zaki et al. 2007, 2012), it is unclear whether a PGPY found to influence blue lupin growth would also affect wheat growth, thus having the potential to influence a break-crop or crop rotation system.

With the above as background, the aim of this study was to establish whether a single yeast strain could be used as a bio-fertiliser for both blue lupin and wheat, by assessing the effect of the yeast strain on the germination, growth and physiology of both plants. To achieve this, the first objective of this study was to isolate a yeast from the rhizosphere of blue lupin and to determine whether this yeast (*Papiliotrema laurentii* CAB 91) could affect the BNF, growth, and nutritional physiology of nodulated blue lupin in the presence and absence of mycorrhizal fungi (Chapter 2). The second objective of this study was to determine the PGP traits of *P. laurentii* CAB 91 and to compare these traits to that of other rhizosphere yeasts. Subsequently, the influence of *P. laurentii* CAB 91 on germination, growth,

photosynthesis and vigour of blue lupin and wheat was investigated in pot trials (Chapter 3). Lastly, some concluding remarks and future challenges with regard to using knowledge of plant-yeast symbioses to develop bio-fertilisers were discussed (Chapter 4).

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LIST OF ABBREVIATIONS

%NDFA	%N derived from atmosphere
ACC	1-aminocyclopropane-1-carboxylic acid
A_{max}	Maximum rate of CO_2 assimilation under light saturating conditions
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BGA	Below-ground allocation
BLK	Blue lupin kernel meal
BLS	Blue lupin seeds
BNF	Biological N_2 fixation
BNF efficiency	Efficiency of N_2 fixation
C	Carbon
C_i	Substomatal carbon dioxide
CK	Cytokinins
CRISPR	Clustered regularly interspaced short palindromic repeats
DF	Dworkin and Foster
DW	Dry weight
E	Transpiration
FW	Fresh weight
GI	Glycaemic index
G_s	Rate of stomatal conductance to carbon dioxide
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IAEA	International Atomic Energy Agency
ICP-AES	Inductively coupled plasma atomic emission spectrometry

IRMS	Isotope ratio mass spectrometry
ITS	Internal transcribed spacer
IWUE	Intrinsic water-use efficiency
JA	Jasmonic acid
K	Potassium
LAD	Long Ashton decarboxylase
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
MANOVA	Multivariate analysis of variance
MGT	Mean germination time
MS	Murashige and Skoog
MSG	Maguire's Speed of Germination
N	Nitrogen
N_2	Atmospheric di-nitrogen
N_{atm}	Nitrogen that originated from the atmosphere through fixation
NBRIP	National Botanical Research Institute's phosphate growth medium
N_{sand}	Nitrogen that was taken up from the sand via the roots
O-CAS	Overlay chrome azurol S
P	Phosphorus
PAs	Polyamines
PCR	Polymerase chain reaction
PGP	Plant growth promoting
PGPM	Plant growth promoting microorganisms
PGPR	Plant growth promoting rhizobacteria
PGPY	Plant growth promoting yeasts
PO_4^{3-}	Phosphate

Pp	Potential productivity
PSS	Physiological saline solution
PVK	Pikovskaya's medium
PWUE	Photosynthetic water-use efficiency
Rd	Leaf dark respiration
RFLP	Restriction fragment length polymorphism (RFLP)
RGR	Relative growth rate
RPM	Revolutions per minute
SE	Standard error
SNAR	Specific nitrogen absorption rate
$SNAR_{Atm}$	Nitrogen absorption rate from the atmosphere through fixation
$SNAR_{sand}$	Rate of nitrogen absorption from sand
SNUR	Specific nitrogen utilisation rate
TMV	Thymine-mineral-vitamin
TSB	Tryptone soy broth
UN	United Nations
V-PDB	Vienna Pee-Dee Belemnite
YM	Yeast extract-malt extract
YM_c	Yeast extract-malt extract supplemented with 0.2% (w/v) chloramphenicol
Zn	Zinc

Chapter 1 – Literature Review

1. The impact of climate change on agricultural production

In the 2014 release of the United Nations' (UN) projections, it was estimated that the world population will reach 9.60 billion people by 2050 (FAO and ITPS 2015). To meet the global food demand in 2050, agricultural production must escalate by 60% (FAO 2016). This translates to an increase from 2.1 billion tonnes to 3 billion tonnes for cereal production alone (Alexandratos and Bruinsma 2012). Similarly, yields of other crops, as well as livestock production, will also have to increase by 2050 if the population's demands are to be met. Although it is possible to escalate yields on a global scale, climate change is predicted to have several negative impacts on agriculture (FAO and ITPS 2015), which will severely reduce agricultural output by 2030 (FAO 2016). Climate change will result in extreme weather events, such as drought and flooding, as well as increases in maximum temperatures. It is estimated that maximum temperatures will rise with 1.8˚C to 4.0˚C by 2100 if climate change is not mitigated (FAO 2009b). Considering that yields of *Triticum aestivum* L. (wheat) and *Zea mays* L. (maize) were found to decline due to increased temperatures (Gregory et al. 1999), it is expected that cereal yields will decrease as a consequence of climate change. Furthermore, some countries that are already experiencing food insecurity are those that will face the greatest increase in maximum temperatures, which will enlarge the numbers of undernourished individuals in these areas (FAO et al. 2015).

Climate change will also have an impact on food production in both irrigated and rainfed areas, since water availability will alter as precipitation pattern and quantity change (Turral et al. 2011). Additionally, higher temperatures will result in greater evaporation, which will be problematic in arid regions (FAO and ITPS 2015). Considering that an increasing number of countries are reaching alarming levels of water scarcity (FAO 2009a) and that this is likely to worsen as climate change continues, it is apparent that food security in arid regions are severely threatened (Lipper et al. 2014). Furthermore, it is expected that global warming will increase the frequency and intensity of floods in subtropical areas (FAO 2011), thereby reducing crop production (FAO 2016), while degrading arable land. Alarmingly, availability of arable land is already declining irrespective of climate change (Gibbs and Salmon 2015) because of biodiversity loss, compaction, contamination, erosion, landslides, organic matter decline, and salinization (Godfray et al. 2010; Gardi et al. 2015).

Therefore, it is expected that further degradation of arable land brought about by climate change will severely reduce agricultural output.

In addition to the concerns mentioned above, it is predicted that climate change will result in increased weeds (FAO 2009a), thus reducing agricultural production. For example, it is anticipated that an increase in the incidence of weeds brought about by climate change will limit pasture productivity in Australia. Climate change will also drive the emergence of new pests and agricultural diseases (FAO 2016), particularly that of plants, since abiotic stresses brought on by climate change will alter host susceptibility to emerging plant pathogens (Fones and Gurr 2017). It is thus expected that emerging plant pathogens will present a threat to food security in several regions as a result of climate change (Fisher et al. 2012). Due to all of the above-mentioned factors, food availability will be reduced by climate change, thereby exposing both rural and urban poor to fluctuating and higher food prices (FAO 2016). To prevent this disaster, agricultural production needs to increase in a sustainable manner, while taking into account to potential threat of climate change.

2. Increasing agricultural production

Currently there are three main means to increase agricultural production, *i.e.* expansion of land, heightened cropping intensity and increasing yields. It is estimated that in developed countries approximately 10% of production increases (20% in developing countries) will stem from expansion of arable land (FAO 2009a). Therefore, 90% of production growth in developed countries, and 80% in developing countries, need to be the result of heightened intensification, *i.e.* increased cropping intensity and yields (Bruinsma 2009).

Although the theoretical production limits under different conditions are contested for major crops (Godfray et al. 2010), it is accepted that there is scope to escalate crop output (Foley et al. 2011). While the major goal is to continuously increase crop yields (Godfray et al. 2010), climate change necessitates that these increases are coupled to nutrient and water use efficiency, together with tolerance to abiotic stress. This can be achieved by employing practices such as the use of nitrogen (N) efficient and heat-tolerant crop varieties, as well as water-saving strategies (FAO 2009a, 2016). In addition, owing to the negative effects of chemical fertilisers on the environment (Vitousek et al. 1997; Smil 2000; Adesemoye and Kloepper 2009), it has been

suggested that natural biological processes should be harnessed to improve soil fertility (FAO 2009a). Considering that many yield gaps are due to insufficient nutrient availability (Foley et al. 2011) and that these gaps are located in countries where the use of chemical fertilisers is chiefly absent, it is imperative that other sustainable methods are employed to escalate crop yields. A well-known method to increase yield sustainably is the employment of break-crops and crop rotation systems (Angus et al. 2015), which are commonly implemented on land used for cereal production. The cultivation of wheat in developed countries has benefitted majorly from such practices, thus rendering the usage of break-crops and crop rotation systems an attractive strategy to increase wheat yield in developing countries. Before the role of break-crops and crop rotation in wheat cultivation can be reviewed, however, it is essential to discuss wheat and its importance as feed and food.

3. Wheat

Wheat (Fig. 1.1) is an annual plant (DuPont et al. 2014; Juhász et al. 2015) that belongs to the family Poaceae, subfamily Pooideae and tribe Triticeae (Soreng et al. 2015). Although other cereals such as *Hordeum vulgare* L. (barley) and *Secale cereale* L. (rye) are also classified into the same tribe as wheat (Middleton et al. 2014; Soreng et al. 2015; Wenxuan Liu et al. 2016), the latter is the only domesticated species belonging to the subtribe Triticinae (Soreng et al. 2015). Wheat is one of the oldest cultivated grains (Bharath Kumar and Prabhasankar 2014) and the second most produced grain crop in the world (FAO 2017). It is cultivated in almost every country around the globe (Table 1.1) to such an extent that the total harvested grain amounted to *ca.* 749 million tons in 2016 (FAO 2017). The highest production during this season occurred in Australia, Canada, China (mainland), France, Germany, India, Pakistan, Russian Federation, Ukraine, and the United States of America. Together these countries contributed to approximately 70% (*ca.* 520 million tons) of the world's wheat production in the 2016 season. It is estimated that approximately 65% of the globally harvested grain is used as food, 17% as animal feed and 12% for industrial applications (FAO 2013).

Figure 1.1. Photograph of one-month old *Triticum aestivum* L., cv. SST 047 (wheat) which was cultivated in silica sand

Table 1.1. Summary of the area under wheat production in million ha (Harvested area), amount of wheat produced in million tonnes (Production) and wheat yield in tonnes ha^{-1} (Yield) for each country as reported for 2016.

Country	Harvested area [a]	Production [a]	Yield [b]
Afghanistan	2.3002	4.5551	1.98
Albania	0.0705	0.2750	3.90
Algeria	1.4428	2.4401	1.69
Angola	0.0055	0.0040	0.73
Argentina	5.6292	18.5575	3.30
Armenia	0.1080	0.3504	3.24
Australia	11.2822	22.2745	1.97
Austria	0.3151	1.9704	6.25
Azerbaijan	0.5906	1.7999	3.05
Bangladesh	0.4450	1.3482	3.03
Belarus	0.7104	2.3400	3.29
Belgium	0.2063	1.4001	6.79
Bhutan	0.0015	0.0027	1.83
Bolivia (Plurinational State of)	0.2418	0.3462	1.43
Bosnia and Herzegovina	0.0714	0.3066	4.29
Brazil	2.1662	6.8344	3.16
Bulgaria	1.1926	5.6627	4.75
Burundi	0.0100	0.0068	0.68
Cameroon	0.0007	0.0009	1.33
Canada	9.2616	30.4867	3.29
Chad	0.0009	0.0017	2.00
Chile	0.2853	1.7319	6.07

Table 1.1. continued

Country	Harvested area [a]	Production [a]	Yield [b]
China (mainland)	24.3458	131.6890	5.41
China (Taiwan Province of)	0.0026	0.0074	2.81
Colombia	0.0017	0.0027	1.61
Croatia	0.1680	0.9601	5.71
Cyprus	0.0091	0.0228	2.51
Czechia	0.8397	5.4547	6.50
Democratic People's Republic of Korea	0.0369	0.0567	1.54
Democratic Republic of the Congo	0.0062	0.0082	1.32
Denmark	0.5830	4.2015	7.21
Ecuador	0.0044	0.0067	1.53
Egypt	1.3688	9.0000	6.58
Eritrea	0.0254	0.0313	1.23
Estonia	0.1645	0.4555	2.77
Ethiopia	1.6961	4.5379	2.68
Finland	0.2151	0.8239	3.83
France	5.5626	29.5045	5.30
Georgia	0.0492	0.1266	2.57
Germany	3.2017	24.4638	7.64
Greece	0.6129	1.6980	2.77
Guatemala	0.0003	0.0007	2.13
Honduras	0.0022	0.0012	0.56
Hungary	1.0556	4.7880	4.54
India	30.2300	93.5000	3.09

Table 1.1. continued

Country	Harvested area [a]	Production [a]	Yield [b]
Iran (Islamic Republic of)	5.6818	11.0976	1.95
Iraq	0.9201	3.0529	3.32
Ireland	0.0679	0.6477	9.54
Israel	0.0437	0.1680	3.84
Italy	1.9124	8.0379	4.20
Japan	0.2144	0.7908	3.69
Jordan	0.0278	0.0312	1.12
Kazakhstan	12.3735	14.9854	1.21
Kenya	0.1531	0.2224	1.45
Kuwait	0.0000	0.0000	5.00
Kyrgyzstan	0.2704	0.6615	2.45
Latvia	0.4791	2.0623	4.30
Lebanon	0.0448	0.1477	3.30
Lesotho	0.0046	0.0040	0.87
Libya	0.2071	0.1646	0.79
Lithuania	0.8709	3.7984	4.36
Luxembourg	0.0138	0.0701	5.07
Madagascar	0.0021	0.0050	2.44
Malawi	0.0007	0.0008	1.11
Mali	0.0045	0.0401	8.93
Malta	0.0033	0.0160	4.84
Mauritania	0.0036	0.0077	2.15
Mexico	0.7236	3.8629	5.34
Mongolia	0.3551	0.4671	1.32

Table 1.1. continued

Country	Harvested area [a]	Production [a]	Yield [b]
Montenegro	0.0007	0.0024	3.15
Morocco	2.4136	2.7311	1.13
Mozambique	0.0156	0.0171	1.09
Myanmar	0.0865	0.1026	1.19
Namibia	0.0022	0.0139	6.33
Nepal	0.7458	1.7368	2.33
Netherlands	0.1273	1.0165	7.98
New Caledonia	0.0000	0.0000	4.00
New Zealand	0.0499	0.4593	9.20
Niger	0.0041	0.0082	2.01
Nigeria	0.0600	0.0600	1.00
Norway	0.0668	0.3088	4.62
Occupied Palestinian Territory	0.0174	0.0330	1.90
Oman	0.0009	0.0028	2.99
Pakistan	9.1431	26.0052	2.84
Paraguay	0.5200	1.1440	2.20
Peru	0.1272	0.1911	1.50
Poland	2.3841	10.8279	4.54
Portugal	0.0382	0.0900	2.36
Qatar	0.00003	0.00006	2.00
Republic of Korea	0.0094	0.0288	3.07
Republic of Moldova	0.3708	1.2929	3.49
Romania	2.1353	8.4311	3.95
Russian Federation	27.3128	73.2946	2.68

Table 1.1. continued

Country	**Harvested area** [a]	**Production** [a]	**Yield** [b]
Rwanda	0.0364	0.0837	2.30
Saudi Arabia	0.1222	0.7658	6.27
Serbia	0.5951	2.8845	4.85
Slovakia	0.4166	2.4342	5.84
Slovenia	0.0315	0.1631	5.19
Somalia	0.0026	0.0010	0.40
South Africa	0.5084	1.9095	3.76
Spain	2.0781	6.4339	3.10
Sudan	0.2167	0.5160	2.38
Swaziland	0.0003	0.0006	1.74
Sweden	0.4485	2.8345	6.32
Switzerland	0.0885	0.3867	4.37
Syrian Arab Republic	1.3151	2.9368	2.23
Tajikistan	0.2975	0.9171	3.08
Thailand	0.0013	0.0013	1.00
The former Yugoslav Republic of Macedonia	0.0798	0.3064	3.84
Tunisia	0.5084	0.9265	1.82
Turkey	7.6099	20.6000	2.71
Turkmenistan	1.4772	1.6000	1.08
Uganda	0.0145	0.0230	1.58
Ukraine	6.2058	26.0988	4.21
United Arab Emirates	0.00002	0.00008	3.73
United Kingdom	1.8230	14.3830	7.89

Table 1.1. continued

Country	Harvested area [a]	Production [a]	Yield [b]
United Republic of Tanzania	0.1027	0.0961	0.94
United States of America	17.7618	62.8591	3.54
Uruguay	0.2150	0.7570	3.52
Uzbekistan	1.4463	6.9405	4.80
Venezuela (Bolivarian Republic of)	0.0000	0.0001	2.98
Yemen	0.1268	0.2202	1.74
Zambia	0.0242	0.1595	6.60
Zimbabwe	0.0237	0.0433	1.83
World average	**220.1076**	**749.4601**	**3.40**

[a] Data obtained from FAOSTAT (available at http://faostat.fao.org/) on 1 February 2018
[b] Yield for each country was calculated by dividing wheat production by the harvested area. Values in red indicate that the yields are less than that of the world average, while values in green show that the yields are higher than that of the world average

3.1. Wheat as food and feed

Wheat grain is widely used as a food source by humans (FAO 2013), mainly in the form of foods produced from flour, *e.g.* biscuits, bread, crackers, muesli and muffins (Shewry 2009). Typically, there are three types of wheat flour, *i.e.* white flour (obtained by milling the endosperm), brown flour (some of the bran and germ is included) and wholemeal flour (made from the entire grain). Although the chemical composition of wheat grain may differ due to variations in environmental and genetic factors (Rosenfelder et al. 2013), the endosperm largely consists of starch (60 to 70%) (Shewry 2009). This high starch content serves as the main energy source when consumed (Zijlstra et al. 1999; Black 2001) and contributes to the high glycaemic index (GI) of wheat based products (Bharath Kumar and Prabhasankar 2014; Edwards et al. 2015; López-Barón et al. 2017). Owing to the high-energy content of wheat grain, it is not only used as food for human consumption, but also as animal feed.

During 2013, approximately 17.8% of the world's wheat grain production (FAO 2015) was allocated towards the rearing of livestock, specifically chickens (Singh et al. 2014;

Amerah 2015), cows (He et al. 2015) and pigs (Rosenfelder et al. 2013; Amerah 2015). In addition to wheat grain, by-products generated during grain milling are used as feed to reduce the costs associated with livestock rearing (Rosenfelder et al. 2013). These by-products constitute up to 25% of the original grain and primarily consist of the pericarp and outermost tissues of the seed, including the aleurone layer and varying amounts of endosperm (Hassan et al. 2008). As an additional amount of harvested grain is utilised when wheat milling by-products are included in animal feed, usage of these products in livestock rearing is of economic value (Jondreville et al. 2000).

It is thus evident that wheat plays an important role in human and animal nutrition, which drives the demand for this crop. To ensure that yields remain high to meet this demand, farmers regularly apply N fertilisers in surfeit to soil during wheat cultivation (Diacono et al. 2013; Kong et al. 2017). Yet, excessive use of N fertilisers is associated with reduced wheat yields, due to greater disease susceptibility (Kong et al. 2017), heightened risk for delayed senescence (Yang et al. 2000, 2004) and lodging (Bundy and Andraski 2004; Hawkesford 2014; Kong et al. 2017), as well as reduced grain filling as a result of disturbed N metabolism (Kong et al. 2017). These negative effects, together with the need to sustainably increase yield, has led to the employment of break-crops and crop rotation to escalate wheat production.

3.2. Effect of break-crops and crop rotation on wheat yield

Several studies have shown that wheat yield is increased in break-crop (Kirkegaard et al. 2008; Angus et al. 2015) and crop rotation (St. Luce et al. 2015) systems. In a break-crop system a single alternative crop is grown before a cereal crop (Angus et al. 2015), whereas crop rotation entails the cultivation of different crops in a systematic and recurring sequence in the same field (Liebman and Dyck 1993). Since leguminous plants are able to fix di-nitrogen (N_2), they are often used in these systems to reduce N fertiliser input (O'Donovan et al. 2014; Preissel et al. 2015; St. Luce et al. 2015) without decreasing wheat yield (Brisson et al. 2010; Preissel et al. 2015). Additionally, wheat yields are increased when grown in a field previously planted with legumes, compared to yields obtained during successive cultivation of wheat (Angus et al. 2015). This phenomenon has not only been ascribed to greater soil N content as a result of biological N_2 fixation (BNF) associated with legumes (Walley et al. 2007; Peoples et al. 2009; Angus et al. 2015), but also to non-N benefits, such as growth of

beneficial soil microorganisms (Biederbeck et al. 2005; Kirkegaard et al. 2008; Tiemann et al. 2015), increased water availability (Kirkegaard et al. 2008; Cutforth et al. 2013) and reduction in disease (Kirkegaard et al. 2008; Angus et al. 2015).

Lupins are often used in break-crop and crop rotation systems (Hane et al. 2017), since these plans can grow in nutrient poor soils (Lambers et al. 2013). One such a lupin is *Lupinus angustifolius* L. (blue lupin; Fig. 1.2), which survives in low nutrient soils by forming a symbiotic association with *Bradyrhizobium lupini* (Peix et al. 2015), resulting in this plant attaining of N via BNF (Kim and Rees 1994; Zahran 1999), as well as by exuding carboxylates from its roots to acquire phosphorous (P) from the soil (Egle et al. 2003; Lambers et al. 2013). In the 'grain belt' of Western Australia (Berger et al. 2013), blue lupin forms an important component of crop rotations (Nuruzzaman et al. 2005; Lambers et al. 2013), since this plant increases wheat yield (Reeves et al. 1984; Doyle et al. 1988; Evans et al. 1991; Williams et al. 2014; Angus et al. 2015) by raising soil N content (Reeves et al. 1984; Martínez-Villaluenga et al. 2006; Pearse et al. 2006), while reducing disease (Reeves et al. 1984). Consequently, Australia had the greatest area under lupin cultivation in 2014 (Table 1.2). In addition to use in Australia, blue lupin is employed in crop rotation systems in Mediterranean climates (Jones 2001; McNeill and Fillery 2008; Wijayanto et al. 2009) and serves as a pulse crop in organic farming systems in Eastern Europe, North Africa and South America (Sujak et al. 2006; Gulewicz et al. 2008). Furthermore, seeds of this plant can serve as food for humans and animals, thereby increasing the agricultural value of blue lupin.

Table 1.2. Summary of the area under lupin production in thousand ha (Harvested area), amount of lupin produced in thousand tonnes (Production) and lupin yield in tonnes/ ha (Yield) for each country as reported for 2016.

Country	Harvested area [a]	Production [a]	Yield [b]
Argentina	0.111	0.166	1.50
Australia	534.084	651.946	1.22
Austria	0.145	0.249	1.72
Belarus	7.378	14.950	2.03
Chile	13.255	27.507	2.08

Table 1.2. continued

Country	Harvested area [a]	Production [a]	Yield [b]
Ecuador	3.725	1.345	0.36
Egypt	0.327	0.679	2.08
France	7.702	16.519	2.14
Germany	28.600	50.000	1.75
Greece	0.429	0.620	1.45
Hungary	0.127	0.105	0.83
Italy	3.385	4.735	1.40
Latvia	0.200	0.300	1.50
Lebanon	0.061	0.134	2.20
Lithuania	3.773	4.564	1.21
Morocco	91.859	61.631	0.67
Peru	11.115	14.019	1.26
Poland	130.064	206.247	1.59
Portugal	0.007	0.007	1.00
Russian Federation	117.395	184.679	1.57
Slovakia	0.624	1.026	1.64
South Africa	10.132	9.516	0.94
Spain	3.886	2.453	0.63
Switzerland	0.115	0.224	1.95
Syrian Arab Republic	0.010	0.011	1.10
Ukraine	18.700	31.210	1.67
World average	**987.208**	**1284.843**	**1.30**

[a] Data obtained from FAOSTAT (available at http://faostat.fao.org/) on 1 February 2018
[b] Yield for each country was calculated by dividing lupin production by the harvested area. Values in red indicate that the yields are lower than that of the world average, while values in green show that the yields are greater than the world average

4. Blue lupin as food and feed

Blue lupin (Fig. 1.2) is an annual grain legume characterised by lateral branches, palmate leaves (Erdemoglu et al. 2007) and the formation of blue, pink or white flowers. It is classified into the 'Old World' lupin group, which comprises 12 lupin species that is distributed around the Mediterranean and North Africa (Susek et al. 2017). Blue lupin together with three other domesticated species, *i.e.* the 'Old World' lupins *Lupinus albus* L. (white lupin) and *Lupinus luteus* L. (yellow lupin), as well as the 'New World' lupin *Lupinus mutabilis* L. (pearl lupin) (Foley et al. 2015), are known as sweet lupins because of their lower alkaloid content (Martínez-Villaluenga et al. 2006) in comparison to the bitter varieties. Yet, blue lupin is cultivated in preference to the other three sweet lupins (Clements et al. 2014), due to its high grain yield (Clements et al. 2014; Kroc et al. 2014) and low seed alkaloid content, thus rendering it favourable for human and animal consumption.

Figure 1.2. Photograph of *Lupinus angustifolius* L., cv. Gunyidi (blue lupin) cultivated in silica sand for one month.

The use of blue lupin seeds (BLS) has increased in the food industry ever since some research groups demonstrated that consumption of these seeds holds several benefits for human health. These benefits include appetite suppression (Lee et al. 2006; Hodgson et al. 2010), improved bowel functioning (Johnson et al. 2006) and cardiovascular health (Belski et al. 2011; Thambiraj et al. 2015), lowered blood glucose (Hall et al. 2005; Bertoglio et al. 2011) and cholesterol levels (Hall et al. 2005; Martins et al. 2005), reduced risk to develop colon cancer (Stephany et al. 2016), as well as stimulation of colon microbiota (Thambiraj et al. 2015). Therefore, flour prepared from BLS has been incorporated into several food products, *e.g.* biscuits (Jayasena and Nasar-Abbas 2011), bread (Clark and Johnson 2002; Villarino et al. 2016), instant noodles (Jayasena et al. 2010b), pasta (Clark and Johnson 2002; Jayasena and Nasar-Abbas 2012) and tofu (Jayasena et al. 2010a). Addition of this flour to products with high GI values may not only improve the product's protein and dietary fibre contents (Hall and Johnson 2004; Belski et al. 2011; Villarino et al. 2015), but can also reduce the GI value of the product (Hall et al. 2005). Considering these benefits and that BLS was shown to have a high dietary fibre (25 to 30%) and protein content (40 to 45%) (Stephany et al. 2016; Hane et al. 2017), it is not surprising that BLS are now considered to be a human health food.

As a result of the high protein content of BLS, blue lupin kernel meal (BLK) prepared from dehusked BLS, can be used in animal feed as an alternative to soybean meal (Batterham 1979; Lestingi et al. 2015). This is desirable in sustainable agriculture, since the price of soybean meal contributes significantly to the rearing cost of livestock (Lanza et al. 2011; Scerra et al. 2011). Yet, anti-nutritive factors of BLS, such as high levels of non-starch polysaccharides and oligosaccharides, are impeding the use of BLK as a complete substitute to soybean meal in animal feed (Drażbo et al. 2014). Therefore, current research focuses on the effect of BLK supplementation in feed on the nutrition of chickens (Nalle et al. 2011; Drażbo et al. 2014; Lee et al. 2016), pigs (Kasprowicz-Potocka et al. 2016; Pieper et al. 2016) and sheep (Ephrem et al. 2015; Sopková et al. 2016). While the use of BLK in the rearing of livestock is still under contention, its use in aquaculture is well established.

Usually feed used in aquaculture is supplemented with fish meal based on its high protein content, well-balanced amino acid profile and good digestibility (Gatlin et al.

2007). Owing to the rapid expansion of aquaculture (Olsen and Hasan 2012), the supply of fish meal became unstable, which resulted in the need for a substitute. Since it was demonstrated that BLK provides adequate nutrition to several aquaculture species (Burel et al. 1998, 2000; Carter and Hauler 2000; Glencross et al. 2003, 2007, 2008, 2011; Glencross and Hawkins 2004; Glencross 2006; Katersky and Carter 2009; Salini and Adams 2014), it is often included in fish feed as a substitute to fish meal.

From the preceding discussion, it is evident that the value of blue lupin stretches beyond its use in break-crop and crop rotation systems to increase wheat yields. Although production of blue lupin is lower than that of wheat, usage of blue lupin as a food source is rising. Therefore, the production of both plants will have to escalate to meet the future demand of a growing population. Considering that blue lupin and wheat yield of most production countries are below that of the world average (Tables 1.1 and 1.2), strategies to sustainably increase the yield of both plants must be employed. One promising strategy to improve crop yields is the utilisation of plant growth promoting microorganisms as bio-fertilisers.

5. Plant growth promoting microorganisms (PGPM) as bio-fertilisers

During the past two decades, the usage of commercially available bio-fertilisers has been gaining popularity in the agricultural sector (Timmusk et al. 2017), due to the potential of these preparations to reduce the reliance on chemical fertilisers, while increasing plant growth. In general, bio-fertilisation encompasses the addition of agriculturally useful microbial populations, such as plant growth promoting rhizobacteria (PGPR), mycorrhizal fungi and yeasts, to soils (Bhardwaj et al. 2014).

5.1. Plant growth promoting rhizobacteria as PGPM

It is well known that PGPR can increase growth of a wide range of plants (Vessey 2003; Nadeem et al. 2014), including that of wheat. To date, numerous PGPR strains across different bacterial genera were shown to influence germination, growth, nutrition, photosynthesis, stress response and yield of this plant (recently reviewed in Çakmakçı et al. 2017). In contrast, less is known about the effect of PGPR on growth of blue lupin, with only one study reporting on the ability of the nodule inhabitant *Micromonospora lupini* (Trujillo et al. 2014) to increase blue lupin growth, most likely by contributing to N_2 fixation within the nodules (Trujillo et al. 2010, 2015). Growth

promotion by PGPR is, however, not limited to N_2 fixation and may happen via other direct, as well as indirect, mechanisms (Vessey 2003; Lugtenberg and Kamilova 2009; Parray et al. 2016).

Various direct mechanisms of plant growth promotion, other than N_2 fixation, have been identified for PGPR, *e.g.* P solubilisation (Lipton et al. 1987; Vassilev et al. 2006; Rodríguez et al. 2007) and the production of growth regulators. The latter may include phytohormones, such as indole-3-acetic acid (IAA) (Loper and Schroth 1986; Lambrecht et al. 2000; Khalid et al. 2004; Cassán et al. 2014) and gibberellins (Dobbelaere et al. 2003), stress regulators (*e.g.* 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase) (Glick et al. 1998; Penrose and Glick 2003; Glick 2005, 2014) and other metabolites, *e.g.* polyamines (PAs) (Thuler et al. 2003; Cassán et al. 2009). In contrast, metabolites that inhibit the activity of phytopathogens, such as antibiotics (Chin-A-Woeng et al. 1998; Haas and Keel 2003; Pliego et al. 2008) and siderophores (Kloepper et al. 1980; Schippers et al. 1987), are associated with indirect effects exerted on plant growth by PGPR. Yet, as mentioned earlier, PGPR is not the only PGPM used in commercial bio-fertilisers, as the benefit of mycorrhizal fungi for plant growth is well-known.

5.2. Mycorrhizal fungi as PGPM

Mycorrhizal fungi form symbiotic associations with *ca.* 80 to 85 % of all terrestrial plant species (Abdel-Fattah 2001; Schalamuk et al. 2006; Baum et al. 2015). These fungal symbionts are grouped into four mycorrhizal types, *i.e.* arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi, ericoid mycorrhizal fungi and orchid mycorrhizal fungi (Balestrini and Lumini 2017). Of these types, AMF is the most widespread, contains the most ancient lineages (Lehnert et al. 2017) and forms symbioses with several trees, most herbs and grasses, including wheat (Pellegrino et al. 2015; Balestrini and Lumini 2017). The effect of AMF fungi on wheat is well-known and was demonstrated to result in greater plant biomass, heightened grain yield, higher shoot P content, improved P, N and Zinc (Zn) uptake (recently reviewed by Pellegrino et al. 2015), as well as in increased stress response (Khalafallah and Abo-Ghalia 2008). In contrast to wheat, the genus *Lupinus* is regarded to be non-mycorrhizal (Oba et al. 2001; Sprent and James 2007). Yet it was shown that some lupins can be colonised by these mycobionts (Trinick 1977; Allen et al. 1984; O'Dell and Trappe 1992). In addition, it

was found that wild blue lupin is weakly mycorrhizal (Trinick 1977), however, these plants do not benefit from the association on a nutritional basis (Lambers and Teste 2013).

In general, plants benefit from forming symbioses with mycorrhizal fungi, since the extra-radical hyphae of the mycobiont extend beyond the plants' root depletion zone, thereby acquiring nutrients, such as N and P, for the host plant (Vierheilig et al. 1998; Göllner et al. 2005; Boby et al. 2007, 2008; Lambers and Teste 2013). In return, the fungus receives photosynthetic carbon (C) (Vierheilig et al. 1998; Cavagnaro et al. 2003; Göllner et al. 2005) and lipids (Luginbuehl et al. 2017) from the host plant. Mycorrhizal fungi can also indirectly improve the growth of a host plant by increasing the plant's resistance to phytopathogens (Azcón-Aguilar et al. 2002; Gosling et al. 2006; Willis et al. 2013; Nadeem et al. 2014) or by improving the plant's tolerance to drought and salinity (Marulanda et al. 2006, 2009; Gamalero et al. 2009; Nadeem et al. 2014). Plant growth promotion is, however, not limited to PGPR and mycorrhizal fungi, as yeasts have also been found to serve as PGPM.

5.3. Yeasts as PGPM

During the past two decades, evidence on the ability of yeasts to promote plant growth (Table 1.3) has been increasing steadily. Some of the yeast species listed in Table 1.3 have also been found to improve the nutrition and photosynthesis of their host plants. Yeasts are also able to improve seed germination in a wide range of plants, which includes both dicotyledonous and monocotyledonous species, *i.e. Beta vulgaris* L. (sugar beet; Shalaby and El-Nady 2008), *Brassica juncea* (L.) Czern. (leaf mustard; Ramos-Garza et al. 2016), *Brassica rapa* L. subsp. *pekinensis* (Chinese cabbage; Nakayan et al. 2013), *Cucumis sativus* L. (cucumber; Akhtyamova and Sattarova 2013), *Gossypium hirsutum* L. (cotton; Akhtyamova and Sattarova 2013), *Oryza sativa* L. (rice; Matić et al. 2014), *Vicia faba* L. (faba bean; Gaballah and Gomaa 2004) and *Zea mays* L. (maize; New et al. 2013).

Table 1.3. A list of yeasts capable of plant growth promotion, their effect on plant nutrition (Pn) and photosynthesis (Ph), as well as the plant growth promoting (PGP) trait(s) of each yeast.

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
Candida blankii	*Vicia faba* L. (Faba bean)	Increased shoot growth	Increased leaf K and N	Auxin and gibberellins production; P sol.	(Morsy 2015)*
Candida middelhoveniana	*V. faba* (Faba bean)	Increased shoot growth	Increased leaf K and N	Auxin and gibberellins production; P sol.	(Morsy 2015)*
Candida parapsilosis	*Trifolium repens* L. (White clover)	Increased root and shoot growth	Increased total K, N and P	IAA	(Azcon et al. 2010)
Candida sp.	*Vigna radiata* (L.) R. Wilczek (Mung bean)	Increased plant growth	N.d	IAA; P sol.; siderophore prod.	(Silambarasan and Vangnai 2017)
Candida tropicalis	*Oryza sativa* L. (Rice)	Increased root growth	N.d	ACC deaminase; IAA; PAs; P sol.	(Amprayn et al. 2012)
C. tropicalis	*Prunus armeniaca* L. (Apricot)	Increased shoot growth	Increased leaf K and N	N.d	(Stino et al. 2009) *

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
C. tropicalis	*Sesamum indicum* L. (Sesame)	Increased plant growth	N.d	N.d	(Mukherjee and Sen 2017)
C. tropicalis	*Triticum aestivum* L. (Wheat)	Increased shoot height and growth	N.d	N.d	(Zaki et al. 2007, 2012)
C. tropicalis	*Zea mays* L. (Maize)	Increased plant growth	N.d	IAA; P and Zn sol.	(Mukherjee and Sen 2014)
Cryptococcus sp.	*Brassica oleracea* var. *alboglabra* (L.H.Bailey) Musil (Chinese kale)	Increased plant growth	N.d	ACC deaminase; IAA	(Deng et al. 2012)
Cryptococcus sp.	*Sedum plumbizincicola X.H. Guo et S.B. Zhou ex L.H. Wu*	Increased plant growth	N.d	ACC deaminase (putative); P sol.	(Wuxing Liu et al. 2016)
Cyberlindnera saturnus [d]	*Z. mays* (Maize)	Increased root and shoot growth	N.d	IAA	(Nassar et al. 2005)

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
Debaryomyces hansenii [e]	*Tagetes erecta* L. (Marigold)	Increased plant growth	N.d	IAA	(Azzam et al. 2012)
Meyerozyma caribbica	*Lactuca sativa* L. (Sword leaf lettuce)	Increased plant growth	Increased total K, N and P	IAA; P sol.	(Nakayan et al. 2013)
M. caribbica	*Z. mays* (Maize)	Increased plant growth	Increased total K	IAA; P sol.	(Nakayan et al. 2013)
Meyerozyma guilliermondii	*L. sativa* (Sword leaf lettuce)	Increased plant growth	Increased total K and N	IAA; P sol.	(Nakayan et al. 2013)
M. guilliermondii	*Z. mays* (Maize)	Increased plant growth	Lowered total K and N	IAA; P sol.	(Nakayan et al. 2013)
Papiliotrema laurentii [f]	*Agathosma betulina (Berg.) Pillans* (Buchu)	Increased plant growth	Increased root Fe, Mn and P; Increased photosynthetic rate and water use efficiency	PAs	(Cloete et al. 2009, 2010)

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
Pichia membranifaciens [g]	*B. vulgaris* (Sugar beet)	Increased root and shoot growth	N.d	N.d	(El-Tarabily 2004)
Pseudozyma aphidis	*Nicotania benthamiana* Domin	Increased shoot length and leaf number	N.d	IAA; P sol.; siderophore prod.	(Fu et al. 2016)
Rhodosporidiobolus ruineniae [h]	*N. benthamiana*	Increased shoot length and leaf number	N.d	IAA; P sol.; siderophore prod.	(Fu et al. 2016)
Rhodotorula glutinis	*B. vulgaris* (Sugar beet)	Increased root and shoot growth	N.d	N.d	(El-Tarabily 2004)
R. glutinis	*V. faba* (Faba bean)	Increased plant growth	Increased total K	N.d	(Gaballah and Gomaa 2004)*
Rhodotorula mucilaginosa	*L. sativa* (Sword leaf lettuce)	Increased plant growth	Increased total K, N and P	IAA; P sol.	(Nakayan et al. 2013)

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
R. mucilaginosa	*V. faba* (Faba bean)	Increased shoot growth	Increased leaf K	Auxin and gibberellins production; P sol.	(Morsy 2015)*
R. mucilaginosa	*Z. mays* (Maize)	Increased plant growth	Increased leaf N	IAA; P sol.	(Nakayan et al. 2013)
Rhodotorula sp.	*Brassica rapa* L. subsp. *pekinensis* (Chinese cabbage)	Increased plant growth	N.d	ACC deaminase; IAA	(Wang et al. 2013)
Rhodotorula sp.	*Brassica oleracea* var. *alboglabra* (L.H.Bailey) Musil (Chinese kale)	Increased plant growth	N.d	ACC deaminase; IAA	(Wang et al. 2013)
Rhodotorula sp.	*Brassica napus* L. (Rapeseed)	Increased plant growth	N.d	ACC deaminase; IAA	(Wang et al. 2013)
Rhodotorula sp.	*O. sativa* (Rice)	Increased root number	N.d	P sol.	(Alonso et al. 2008)

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
Rhodotorula sp.	*Solanum lycopersicum* L. (Tomato)	Increased plant growth	N.d	N.d	(Abd El-Hafez and Shehata 2001)
Rhodotorula sp.	X Triticosecale sp. Wittm. ex A. Camus. (Triticale)	Increased plant growth	N.d	N.d	(Bahr and Gomaa 2002)
Saccharomyces cerevisiae	*Cucumis sativus* L. (Cucumber)	Increased root and shoot growth	N.d	N.d	(Karajeh 2013)
S. cerevisiae	*C. sativus* (Cucumber)	Increased plant growth and yield	Increased total Ca and Mg	N.d	(Kang et al. 2015)
S. cerevisiae	*Glycine max* (L.) Merr. (Soybean)	Increased shoot growth	Increased leaf K, N and P	Gibberellic acid production; IAA	(Morsy et al. 2014)
S. cerevisiae	*Z. mays* (Maize)	Increased root growth	N.d	P sol.	(Hesham and Mohamed 2011)
Sporobolomyces roseus	*T. aestivum* (Wheat)	Increased plant growth	N.d	N.d	(Perondi et al. 1996)

Table 1.3. continued

Yeast species	Plant species [a]	Effect on Plant Growth	Effect on Pn and Ph [b]	PGP Trait(s) [b]	Reference [c]
Trichosporon asahii	*B. vulgaris* (Sugar beet)	Increased root and shoot growth	N.d	N.d	(El-Tarabily 2004)
Yarrowia lipolytica	*Dorycnium pentaphyllum* Scop. (Prostrate canary clover)	Increased root growth	No effect on shoot N	N.d	(Medina et al. 2004)
Unknown yeast	*Lupinus albus* L. (White lupin)	Increased plant growth	N.d	N.d	(Khalil and Ismael 2010)

[a] Known plant common names provided in brackets
[b] Abbreviations: ACC deaminase – 1-aminocyclopropane-1-carboxylic acid deaminase activity; Ca – Calcium; Fe – iron; IAA – production of Indole-3-acetic acid; K – potassium; Mg – magnesium; Mn – manganese; N – nitrogen; N.d – Not determined; PAs – production of polyamines; P sol. – phosphorus solubilisation; Zn Sol. – zinc solubilisation
[c] References marked with * indicates that the manuscript was published in a predatory journal
[d] Synonym: *Williopsis saturnus*
[e] Synonym: *Candida famata*
[f] Synonym: *Cryptococcus laurentii*
[g] Synonym: *Candida valida*
[h] Synonym: *Sporidiobolus ruineniae*

Considering that increased seed germination is linked to increased seedling emergence and plant establishment (Marcos Filho 2015; Finch-Savage and Bassel 2016), it is possible to attain greater yields by increasing seed germination. Yet, germination data might not predict seedling emergence in the field (ISTA 2014; Marcos Filho 2015; Finch-Savage and Bassel 2016), since such experiments provide information on seed germination under controlled conditions (Hampton and Coolbear 1990; Hampton and TeKrony 1995; Finch-Savage and Bassel 2016).

As it is generally accepted that seeds with a high vigour will have a good performance in the field (ISTA 2014), seeds are often subjected to vigour tests. Seeds with a high germination and vigour will have a rapid and uniform germination, resulting in seedlings and plants that are able to withstand environmental stress, as well as in a crop that matures uniformly, thereby increasing harvest efficiency (Bennett et al. 2004). Little is, however, known about the mechanisms whereby yeasts improve seed germination. In contrast, the ability of these unicellular fungi to affect plant growth has been attributed to their possession of several plant growth promoting (PGP) traits.

5.3.1. Plant growth promoting traits of yeasts

Similar to PGPR, the PGP traits of plant growth promoting yeasts (PGPY) may affect plants in a direct or indirect manner. The best-known mechanism whereby PGPY promote plant growth is via production of the auxin IAA (Table 1.3). This phytohormone is involved in different physiological processes, such as cell division, cell enlargement and tissue differentiation (Moore 1989; Lüthen et al. 1999), as well as increasing root exudation (Glick 2014). Moreover, plant roots respond to this hormone by forming adventitious (Torrey 1950; Laskowski et al. 1995; Casimiro et al. 2001) and lateral roots (Finnie and Van Staden 1985), which results in increased biomass of the root system (Patten and Glick 1996). This allows for the expansion of above-ground plant organs, thereby increasing overall plant growth.

Some PGPY may also produce the enzyme ACC deaminase (EC 3.5.99.7) (Table 3). This enzyme catalyses the deamination of the ethylene precursor ACC to form α-ketobutyrate and ammonia (Honma and Shimomura 1978; Penrose and Glick 2003). Production of ACC deaminase by PGPY benefits plants, since cleavage of ACC reduces the levels of ‘stress’ ethylene produced by higher plants under environmental stress conditions (Hyodo 1991; Abeles et al. 1992; Morgan and Drew 1997; Stearns

and Glick 2003), thereby limiting the negative effect of 'stress' ethylene on plant growth (Hyodo 1991; Glick et al. 1998).

In addition to IAA and ACC deaminase production, PGPY can increase plant growth by producing polyamines (PAs) (Table 1.3). These aliphatic polycationic compounds (Tabor and Tabor 1985) are able to regulate plant growth (Evans and Malmberg 1989) and can affect plant developmental processes (Evans and Malmberg 1989; Galston and Sawhney 1990). For example, the exogenous application of PAs to plants stimulate seed germination (Sinska 1988) and plant growth (Jarvis et al. 1983; Geneve and Kester 1991; Krizek et al. 1997; Rajasekaran and Blake 1998; Sharma and Ali 1998; Todorov et al. 1998). Polyamines are present in all living organisms (Miller-Fleming et al. 2015) with the most prevalent being spermine, spermidine and putrescine, while small quantities of cadaverine are also present. During biosynthesis of PAs, arginine and ornithine form putrescine via the enzymes arginine decarboxylase (EC 4.1.1.19) and ornithine decarboxylase (EC 4.1.1.17), respectively (Evans and Malmberg 1989; Couée et al. 2004). Putrescine serves as the precursor for the production of spermidine via the enzyme spermidine synthase (EC 2.5.1.16) (Imai et al. 2004) and spermidine is further metabolized to spermine by spermine synthase (EC 2.5.1.22) (Hamasaki-Katagiri et al. 1998; Imai et al. 2004). Cadaverine is formed from lysine via the enzyme lysine decarboxylase (EC 4.1.1.18) (Bagni et al. 1986; Tomar et al. 2013). Therefore, if one or more of the abovementioned enzymes are present in PGPY, these unicellular fungi might be able to produce PAs, which can result in improved plant growth and seed germination.

Lastly, yeasts can also directly improve plant growth through the solubilisation of nutrients, such as P and Zn (Table 1.3). Phosphorus is an essential macronutrient in plant nutrition, as it forms a major component of nucleic acids, membrane lipids and intermediates of energy metabolism (Shen et al. 2011). Yet, most P in soil is rendered insoluble and thus relatively little is available for plant uptake (Sharma et al. 2013). Therefore, symbiotic yeasts capable of P solubilisation, mostly through organic acid secretion (Vassileva et al. 2000), can improve growth by rendering this essential nutrient available to the host plant. The micronutrient Zn is also essential for plant growth, where it forms part of key processes, *e.g.* enzymatic reactions, carbohydrate metabolism and protein synthesis (Gontia-Mishra et al. 2017). However, similar to P,

Zn has a low solubility in soil (Iqbal et al. 2010). Thus, Zn solubilisation by yeasts may ameliorate Zn deficiency in soil, thereby increasing the availability of this micronutrient for plant uptake.

As mentioned earlier, PGPY can have an indirect impact on plant growth. One such a mechanism is the production of siderophores (Table 1.3). These low molecular weight compounds act as iron scavenging molecules by chelating iron (Neilands 1981) and thus provide siderophore producers with a competitive advantage when the bioavailable concentration of this metal is low (Höfte et al. 1992), thereby reducing phytopathogen growth. Not surprisingly, the antagonism of some yeast species belonging to the genus *Rhodotorula* towards phytopathogens of crop plants and fruits was linked to the ability of these unicellular fungi to produce the siderophore rhodotorulic acid (Calvente et al. 1999, 2001; Sansone et al. 2005). Siderophore producing PGPY may therefore potentially reduce phytopathogen growth and thus increase plant survival, as was demonstrated for PGPR (Kloepper et al. 1980). Additionally, yeasts may improve plant growth indirectly by interacting with other microorganisms present in the rhizosphere, thereby forming tripartite interactions. Such symbioses often involve mycorrhizal fungi or rhizobia as the third symbiont.

5.3.2. Tripartite and quadripartite symbioses involving PGPY and other microorganisms

In contrast to plant-yeast symbioses, little research was conducted to understand tripartite interactions involving plants, yeasts and other rhizosphere microorganisms. The least studied of these interactions are those occurring between legumes, rhizobia and yeasts, since only two studies to date were conducted on these symbioses. In a study conducted by Tuladhar and Subba-Rao (1985), it was found that the yeast *Saccharomyces cerevisiae* improved growth and nodulation of *Trifolium alexandrinum* L. (Egyptian clover). These authors also demonstrated that *S. cerevisiae* can improve nodulation and growth of the legumes *Vigna radiata* (L.) R. Wilczek (mung bean), *Glycine max* (L.) Merr. (soybean), as well as *Leucaena leucocephala* (Lam.) de Wit (white leadtree), by stimulating autochthonous rhizobium populations that occurred naturally in garden soil. In another experiment, the same authors found that growth, grain yield and nodulation of mung bean, soybean, *Vigna mungo* (L.) Hepper (black gram) and

Vigna unguiculata (L.) Walp. (cowpea) were increased when inoculated with *Rhizobium trifolii* and *S. cerevisiae*. Similarly, it was shown that growth, nutrient status and nodulation of *Trifolium repens* L. (white clover) increased in the presence of *Candida parapsilosis* (Azcon et al. 2010), which attributed to the ability of this yeast to produce IAA *in vitro*.

Yeasts may also form tripartite interactions with plants and mycorrhizal fungi. In comparison to rhizobia-yeast-plant symbioses, more is known about interactions between mycorrhizal fungi, yeasts and plants. Dual inoculation of plants with mycorrhizal fungi and yeasts often result in greater mycorrhizal colonization and improved growth of the host plant. For instance, it was demonstrated that growth and AMF colonization of *Trifolium pratense* L. (red clover) was increased when dual inoculated with the yeast *Rhodotorula mucilaginosa* and the AMF *Gigaspora rosea* (Fracchia et al. 2003). Similar results were obtained by the same authors when soybean was dual inoculated with *R. mucilaginosa* and *Funneliformis mosseae* (syn. *Glomus mosseae*). Likewise, soybean growth and colonization by *F. mosseae* was improved when *Kazachstania kunashirensis* (syn. *Saccharomyces kunashirensis*), *Papiliotrema laurentii* (syn. *Cryptococcus laurentii*), or *R. mucilaginosa* was used as the third symbiont (Sampedro et al. 2004). Additionally, Göllner et al. (2006) showed that the shoot biomass of maize could be increased when inoculated with *Rhizophagus intraradices* (syn. *Glomus intraradices*) in conjunction with either *Barnettozyma californica* (syn. *Williopsis californica*) or *Candida sake*.

Tripartite associations between mycorrhizal fungi, yeasts and plants may also result in improved plant nutrient status. For example, dual inoculation of *Solanum lycopersicum* L. (tomato) with *Septoglomus deserticola* (syn. *Glomus deserticola*) and *Yarrowia lipolytica* not only resulted in greater plant growth and root colonization by *S. deserticola,* but also in higher P levels in the tissues of the plant (Vassilev et al. 2001). Furthermore, Boby et al. (2008) demonstrated that dual inoculation of cowpea with *F. mosseae* and different yeasts (*i.e. Cutaneotrichosporon cutaneum* [syn. *Trichosporon cutaneum*], *Metschnikowia pulcherrima*, *P. laurentii, R. mucilaginosa*, *S. cerevisiae*, and *Schwanniomyces occidentalis* [syn. *Debaryomyces occidentalis*]) increased the growth, AMF colonization, as well as the total N and P content, of the plants.

In addition to tripartite symbioses, some PGPY has been found to form quadripartite associations with legumes, rhizobia and mycorrhizal fungi. For instance, Singh et al. (1991) demonstrated that the inoculation of *Cajanus cajan* (L.) Millsp. (pigeon pea), cowpea, mung bean, and white leadtree with *S. cerevisiae* resulted in increased AMF colonisation, nodulation and plant growth. Similarly, it was found that AMF colonization, nodulation and root growth of *Dorycnium pentaphyllum* Scop. (prostrate canary clover) was improved when the plant was grown in the presence of *Y. lipolytica* (Medina et al. 2004). Furthermore, triple inoculation of white clover with AMF, *C. parapsilosis* and rhizobia not only increased AMF colonisation and nodulation, but also the growth and N, P and potassium (K) status of the plants (Azcon et al. 2010).

Although it is evident that soil yeasts partake in quadripartite interactions with different legumes, it is unclear whether PGPY may form a quadripartite symbiosis with blue lupin, rhizobia and mycorrhizal fungi. Considering the use of blue lupin in break-crop and crop rotation systems to fix N_2 for wheat growth, it is crucial that the potential impact of such an association on physiological parameters, *e.g.* BNF and nutritional physiology, of blue lupin is explored to ensure that N_2 fixation occurs at an optimal rate. Furthermore, it is unknown whether a PGPY that is capable of improving the growth and physiology of blue lupin, will also be able to influence wheat growth and its physiology, thereby affecting the whole break-crop or crop rotation system. Despite the knowledge that PGPY can increase seed germination, no investigation to date has been directed towards elucidating the influence of yeasts on the germination or vigour of blue lupin and wheat seeds. Therefore, the overall aim of this study was to investigate the effect of a single yeast strain on the germination, growth and physiology of blue lupin and wheat, to obtain a potential bio-fertiliser for both plants.

6. Research objectives

The first objective of this study was to investigate the effect of mycorrhizal fungi and a rhizosphere yeast on the growth and nutritional physiology of blue lupin in the presence of nodulating bacteria. The second objective of this study was to compare the PGP traits of this rhizosphere yeast (*P. laurentii* CAB 91) to that of other rhizosphere yeasts, whereafter the hypothesis that *P. laurentii* CAB 91 can affect germination, growth, photosynthesis and vigour of blue lupin and wheat was investigated in pot trials.

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Chapter 2 – The role of *Papiliotrema laurentii* and mycorrhizal fungi in the nutritional physiology of *Lupinus angustifolius* L. hosting N_2-fixing nodules

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1. Introduction

The blue lupin, *Lupinus angustifolius* L., is a widely-cultivated leguminous plant (Clements et al. 2014; Kroc et al. 2014) that has gained popularity as a crop over other lupins in the past few years due to its high grain yield and lower seed alkaloid content (Clements et al. 2014). In addition to being cultivated for its protein rich grain, this plant is employed in crop rotation practices with cereals in Mediterranean climates (Jones 2001; McNeill and Fillery 2008; Wijayanto et al. 2009). Similar to other legumes, blue lupins form symbioses with rhizobia, which results in the development of root nodules, thereby allowing biological nitrogen fixation (BNF) to take place (Valentine et al. 2010). It is well known that during BNF, nodule inhabiting bacteria fix atmospheric dinitrogen (N_2) into plant available ammonia (Kim and Rees 1994; Zahran 1999). Yet, a large amount of ATP is needed to drive BNF (Halbleib and Ludden 2000; Dixon and Kahn 2004; Ferguson et al. 2013), which may result in limited plant growth in phosphorous (P) deficient soils (Høgh-Jensen et al. 2002). To survive in these low P soils, legumes belonging to the genus *Lupinus* developed the ability to 'mine' P through exudation of carboxylates from cluster (*Lupinus albus* L.) (Gardner et al. 1982) and cluster-like roots (*Lupinus luteus* L.) (Barbas et al. 1999). Not all members of this genus produce these specialized structures (Lambers et al. 2013), as in the case of *L. angustifolius,* which exudes carboxylates directly from its roots (Egle et al. 2003). Yet, this process seems to be less efficient in acquiring insoluble P, as *L. angustifolius* produces less biomass than *L. albus* (Funayama-Noguchi et al. 2015) and *L. luteus* (Brennan and Bolland 2003) when cultivated in soil with low levels of P.

It is widely known that mycorrhizal fungi can aid plants in obtaining more P from soil. The hyphae of these symbionts cross the root depletion zone thereby procuring P for their host plants (Smith et al. 2003; Boby et al. 2007, 2008; Lambers and Teste 2013). In general, legumes can form symbioses with mycorrhizal fungi (Scheublin et al. 2004; Sprent and James 2007), but the genus *Lupinus* is commonly regarded as non-mycorrhizal (Avio et al. 1990; Vierheilig et al. 1994; Oba et al. 2001; Sprent and James 2007). Regardless, it was shown that wild *L. angustifolius* can be weakly mycorrhizal (Trinick 1977), which might aid these plants in obtaining more P for growth and nodulation (Andrade et al. 1998). However, when mycorrhizal fungi are absent other microorganisms, such as yeasts, may aid plant survival (Botha 2011). These unicellular fungi are known to benefit growth of various plants (Vassileva et al. 2000;

Vassilev et al. 2001; Medina et al. 2004a; Medina et al. 2004b; Nassar et al. 2005; El-Tarabily and Sivasithamparam 2006; Cloete et al. 2009; Cloete et al. 2010; Souchie et al. 2010; Azcon et al. 2010; Ampryan et al. 2012; Azzam et al. 2012; Morsy et al. 2014; Mukherjee and Sen 2014; Morsy 2015) and some soil yeasts may also act as P-acquiring symbionts of plants. For example, it was shown that the plant growth promoting yeast (PGPY) *Papiliotrema laurentii* increased the root P concentration of a plant occurring in the nutrient poor Fynbos ecosystem, *i.e.* buchu (*Agathosma betulina* (Berg.) Pillans) (Cloete et al. 2010). Similarly, it was demonstrated that the rock-phosphate solubilizing yeast *Yarrowia lipolytica* (Vassileva et al. 2000) enhances growth of the shrub, prostrate canary clover (*Dorycnium pentaphyllum* Scop.), while increasing the P concentration of the plant (Medina et al. 2004a; Medina et al. 2004b). Yet, plant growth promotion by yeasts is not limited to P nutrition, because various research groups demonstrated that these microorganisms may also increase the levels of other nutrients, *e.g.* nitrogen (N), in plant tissues (Medina et al. 2004b; El-Tarabily and Sivasithamparam 2006; Cloete et al. 2009; Souchie et al. 2010; Azcon et al. 2010; Azzam et al. 2012; Morsy et al. 2014; Mukherjee and Sen 2014). Furthermore, some researchers have ascribed the positive impact of PGPY on plant growth to the *in vitro* production of hormones, such as the auxin indole-3-acetic acid (IAA) (Nassar et al. 2005; Xin et al. 2009; Amprayn et al. 2012; Limtong and Koowadjanakul 2012). Similarly, yeasts that are capable of *in vitro* production of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase are known to improve plant growth (Amprayn et al. 2012; Deng et al. 2012), since this enzyme cleaves the ethylene gas precursor, ACC, thus reducing the levels of this stress-related hormone (Glick 2014). In addition, PGPY can interact with other root symbionts, such as mycorrhizal fungi (Singh et al. 1991; Vassilev et al. 2001; Fracchia et al. 2003; Sampedro et al. 2004; Medina et al. 2004b; Gollner et al. 2006; Boby et al. 2007, 2008; Alonso et al. 2008; Souchie et al. 2010) and rhizobia (Tuladhar and Subba Rao 1985; Singh et al. 1991; Azcon et al. 2010; Morsy et al. 2014; Morsy 2015), often resulting in increased symbiont colonization and improved plant growth.

Despite the importance of leguminous crops for agriculture, only a few studies were aimed at investigating the effect of yeasts on legumes in the presence of both rhizobia and mycorrhizal fungi (Singh et al. 1991; Medina et al. 2004a; Medina et al. 2004b; Azcon et al. 2010; Morsy 2015). Yet, these research groups failed to explore the effect

of these symbionts on plant physiological parameters, such as BNF efficiency, N_2 fixation and specific nutrient absorption and utilization rates. In addition, it is unclear how yeasts and mycorrhizal fungi, either singularly or in combination, would affect the growth, nutritional physiology and BNF of nodulated lupins. Therefore, this study aimed at elucidating the functional consequences of the presence of a mixed-microbial population in the rhizosphere of a lupin, using pot trails. Although such trials exclude much of the variability of field conditions, it allows for controlled conditions (Knight and Will 1971) wherein the interaction between a plant and its microbial symbionts can be studied. The aim was achieved by investigating the effect of mycorrhizal fungi and a rhizosphere yeast on the growth and nutritional physiology of *L. angustifolius* in the presence of nodulating bacteria.

2. Materials and Methods

2.1. Isolation and identification of yeasts from *L. angustifolius* rhizosphere

Lupinus angustifolius L. cv. Gunyidi plants were cultivated outdoors in a 30 L pot containing *c.* 15 kg of Garden Pro potting soil (Pick n Pay, Cape Town, South Africa). After 6 weeks of growth, the roots of an uprooted plant were added to a 250 mL conical flask containing 30 mL yeast extract-malt extract broth (Yarrow 1998), supplemented with 0.2% (w/v) chloramphenicol (YM_c) (Sigma-Aldrich, St Louis, MO, USA). The conical flask was incubated on an Excella E10 platform shaker (100 revolutions per minute [rpm]; New Brunswick Scientific Co. Inc., Edison, NJ, USA) for 2 d at 26 ˚C. To remove filamentous fungi, the resulting culture was transferred into fresh YM_c broth via filtration through sterile glass wool. The flask was subsequently incubated on a shaker under the same conditions as indicated above. After three successive transfer and incubation steps, the final culture was serially diluted and spread plated onto YM_c agar. After 2 d of incubation at 26 ˚C, the Harrison's disc method (Harrigan and McCance 1976) was used to randomly select yeast colonies from the plates. The resulting isolates were subsequently purified on YM_c agar.

The isolates were classified using restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the ribosomal gene cluster. In brief, the isolates were cultured overnight at 26 ˚C in 10 mL yeast extract-malt extract (YM) broth on a TC-7 tissue culture roller drum (New Brunswick Scientific Co. Inc.) set to 60 rpm. The ITS region of the isolates was amplified through colony

polymerase chain reaction (PCR) using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; Inqaba Biotechnical Industries, Pretoria, South Africa) (White et al. 1990). The 50 µL PCR mixture contained 25 µL of a 2x master mix (Thermo Scientific, Waltham, MA, USA), 2 µL of each primer (10 µmol/ L) and 2 µL of the culture. An Applied Biosystems (Foster City, CA, USA) 2720 thermal cycler was used to perform the amplification process. This process comprised of an initial denaturation at 95°C for 6 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 1 min. A final extension at 72°C for 7 min was also included. To obtain RFLP profiles, the amplified ITS regions were digested with the restriction endonucleases Hin6I, HinfI and MboII according to the manufacturer's specifications (Thermo Scientific). The fragments were separated on a 2% (w/v) agarose gel (Lonza Rockland Inc., Rockland, ME, USA) containing 1% (w/v) ethidium bromide (Sigma-Aldrich). The banding patterns were compared to a Lambda DNA/ HindIII Marker (Thermo Scientific) and a representative of each RFLP profile was identified by analyzing the D1/ D2 domain of the large subunit ribosomal DNA (rDNA).

This was achieved by culturing the selected isolates overnight in YM broth as previously described and the D1/ D2 domain was amplified by colony PCR using the forward primer F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3') and the reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3'; Inqaba Biotechnical Industries) (Fell et al. 2000). The PCR reaction was set up as described previously and amplification was performed in an Applied Biosystems 2720 thermal cycler. The program comprised of an initial denaturation at 94°C for 5 min, 36 cycles composed of denaturation at 95°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 1 min, followed by a final extension of 72°C for 7 min. The D1/ D2 nucleotide sequences of the isolates were obtained using an Applied Biosystems ABI3130xl genetic analyzer, whereafter the sequences were compared to known sequences on the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to identify the isolates. The sequences of the isolates were deposited in this database under the accession numbers KP789311 and KP789312. Working cultures of the isolates were maintained on YM_c agar slants at 23 ˚C. The isolates were stored at -80 ˚C in 15% (v/v) glycerol and were deposited in the culture collection of the Department of Microbiology (Stellenbosch University, South Africa) under the strain numbers CAB 90 and CAB 91.

2.2. Seed germination, yeast inoculation and plant growth

To ensure that the microorganisms used in the inoculums were dominant in the rhizosphere and to decrease the risk of contamination with plant pathogens, while allowing exposure to a diversity of microorganisms from the surroundings, all equipment, growth media, seeds and solutions were sterilized prior to use. Silica sand (600 µm grain size; Agrimark, Stellenbosch, South Africa) was acid washed with 0.1 *M* HCl (Merck, Darmstadt, Germany), rinsed three times with distilled water and dried. The sand was sterilized in 1 L glass jars (Consol, Stellenbosch, South Africa) at 121˚C for 20 min in a Huxley Speedy Autoclave (HL-340, Taipei, Taiwan) and dried in an oven (Memmert, Schwabach, Germany) at 80 ˚C for one week before use. *Lupinus angustifolius* seeds (n = 100; Agricol, Cape Town, South Africa) were surface sterilized by first submerging them in 70% (v/v) ethanol for 60 s, then in 1% (v/v) sodium hypochlorite solution for 40 s, which was followed by two rinsing steps in sterile distilled water. Seeds were allowed to imbibe sterile water overnight and were treated with a *Bradyrhizobium* sp. inoculum according to the manufacturer's specifications (Stimuplant, Zwavelpoort, South Africa). The seeds were germinated in the sterile silica sand for one week, in a well-ventilated glasshouse with an average light/dark relative humidity of 50/80% and temperature of 23/15°C. This glasshouse allowed for a daily exposure of 1000 - 1100 µmol m^2 s^{-1} photosynthetic photon flux densities over a photoperiod of 12 h.

An inoculum of *P. laurentii* CAB 91 was prepared by culturing the yeast in 250 mL conical flasks containing 30 mL YM broth on an Excella E10 platform shaker set at 100 rpm for 2 d at 26 ˚C. Yeast cells were pelleted by centrifugation (3800 **g**; 5 min; 4 ˚C) and rinsed twice with sterile physiological saline solution (PSS). The concentration of the resulting suspension was determined using a Neubauer counting chamber (Marienfeld Superior, Lauda-Königshofen, Germany) and adjusted to log 8 cells mL^{-1}. Seedlings of uniform size (n = 20) were dislodged from the silica sand and their roots were treated with either sterile PSS (n = 10) or with the *P. laurentii* CAB 91 suspension (n = 10). Of the PSS treated seedlings five were used as the control treatment and were planted in pots each containing *c.* 800 g of the same sterile silica sand that was used for seed germination. The mycorrhizal fungi (MF) treatment was obtained by planting the remaining PSS treated seedlings (n = 5) in pots that contained *c.* 800 g sterile silica sand, which was mixed with a commercial inoculum (Mycoroot,

Grahamstown, South Africa) of mycorrhizal fungi (*Claroideoglomus etunicatum* [syn. *Glomus etunicatum*], *Funneliformis mosseae* [syn. *Glomus mosseae*], *Gigaspora gigantea, Rhizophagus clarus* [syn. *Glomus clarum*] and *Paraglomus occultum*) to ensure that the results obtained would be comparable to what would be observed in the field. Five of the seedlings treated with the *P. laurentii* CAB 91 suspension were planted in pots each containing *c.* 800 g sterile silica sand resulting in the yeast treatment (CL). The last treatment (CLMF) was obtained by planting the remainder of yeast treated seedlings (n = 5) in pots that each contained the same sand/mycorrhizal fungi mix as prepared for the MF treatment. All of the plants were cultivated for one month in a glasshouse, at the same conditions described earlier. Each plant received 50 mL of a modified Long Ashton nutrient solution (Cloete et al. 2009), containing a low amount of P (10 µ*M*) (Le Roux et al. 2006; Le Roux et al. 2008; Kleinert et al. 2014; Thuynsma et al 2014a, 2014b; Le Roux et al. 2014) in the form of Na_2HPO_4 and NaH_2PO_4 (Merck), on a weekly basis. In addition, the plants received sterile distilled water as needed.

The initial yeast concentration present on the seedling roots directly after treatment was also determined, by rinsing roots (n = 3) treated with the *P. laurentii* CAB 91 suspension in sterile PSS. The resulting suspensions were serially diluted and spread plated onto YM_c agar. Colonies present on the plates were enumerated after 2 d of incubation at 26 ˚C. In addition, the seedlings were dried in an oven for 1 week at 80 ˚C, after which the dry weight (DW) of the roots and shoots were recorded. The resulting DW was used to standardize yeast numbers present on the roots directly after treatment.

2.3. Harvesting, biomass parameters, mycorrhizal colonization and yeast enumeration

After one month of growth, the *L. angustifolius* plants were harvested, and their roots rinsed in sterile PSS. Subsamples of the roots were collected and stored in 50% (v/v) ethanol until mycorrhizal staining was performed. The plants were divided into roots, shoots and nodules, and dried in an oven for 1 week at 80 ˚C. The DW of the different plant organs was recorded and used to calculate the relative growth rate (RGR) of the roots, shoots, nodules, and whole plant (Sibly and Vincent 1997) by using the DW of the seedlings as the initial values. In addition, the below-ground allocation (BGA),

which represents the fraction of new root biomass partitioned into nodules and new roots over the growth period, was calculated using the equation provided by (Bazzaz 1997). The percentage nodules per symbiotic root, representing the proportion of dry mass allocated to nodules in the below-ground root system, was expressed as

$$\%\text{nodules per symbiotic root} = \left(\frac{X}{Y}\right) \times 100 \quad (1)$$

where *X* represents the nodule mass and *Y* the root mass.

Mycorrhizal colonization was determined by clearing the root subsamples in 10% (w/v) KOH at 90 ˚C for 1.5 h. The roots were rinsed in distilled water and stained in 0.03% (w/v) chlorazol black E (Sigma-Aldrich) in lactoglycerol (lactic acid-glycerol-water, 1:1:1; v/v/v) at 90 ˚C for 1 h. Roots were allowed to destain in sterile 50% (v/v) glycerol for 2 d and examined microscopically for mycorrhizal colonization, which was quantified using the gridline intersect method as described by Giovannetti and Mosse (1980). The number of yeast cells present in the rhizosphere of each *L. angustifolius* plant was enumerated by preparing dilution plates of the PSS wherein the roots were rinsed, using thymine-mineral-vitamin (TMV) agar plates (Cornelissen et al. 2003). Yeast colonies present on the plates were counted after 1 week of incubation at 26 ˚C. Random colonies were selected from the plates and the isolates were identified using RFLP and gene sequence analyses as explained earlier.

2.4. %NDFA and nutritional physiology

The effect of the different treatments on plant physiology was determined by analyzing the carbon (C), N and P content of the plant material, which was used to perform different calculations. The plants were milled for 1 min in 35 mL stainless steel capsules, each containing one 10 mm stainless steel ball, using a Retsch MM 400 mixer mill (Haan, Germany) set at 25 Hz s^{-1}, whereafter *c.* 0.002 g of each sample was set aside for P analysis. The remainder of the samples was analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ using gas-source isotope ratio mass spectrometry (IRMS). For this analysis, the samples were milled even further in a Wiley mill (0.5 mm mesh; Thomas Scientific, Swedesboro, NJ, USA), after which *c.* 0.00215 g of each sample was transferred into 8 mm x 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, United Kingdom) and combusted using a Fisons NA (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The δ-values for the released C and N gasses were determined on a

Finnigan Matt 252 isotope ratio mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) connected to a CHN analyzer by a Finnigan MAT conflo control unit. To correct the samples for machine drift, Merck Gel and Acacia were used as in-house standards, while ammonium sulfate ($[NH_4]_2SO_4$) was used as an International Atomic Energy Agency (IAEA) standard. Isotopic ratios (δ^{13} C and δ^{15} N) were calculated relative to the standards Vienna Pee-Dee Belemnite (V-PDB) for δ^{13} C and atmospheric N_2 for δ^{15} N (Farquhar et al. 1989).

The C and N concentrations obtained from gas-source IRMS were used to calculate the construction cost of new tissue (mmol C g^{-1} DW) according to Kleinert et al. (2014), which was in turn used to calculate the daily growth respiration of the plants (µmol CO_2 d^{-1}) (Peng et al. 1993). In addition, the interaction between the different treatments and nodule function was investigated by calculating the %N derived from atmosphere (%NDFA) using the equation (Shearer and Kohl 1986):

$$\%\text{NDFA} = 100 \times \frac{\delta^{15}N_{ReferencePlant} - \delta^{15}N_{Legume}}{\delta^{15}N_{ReferencePlant} - B\ Value} \quad (2)$$

where *Triticum aestivum* L. served as the reference plant and was cultivated under the same glasshouse conditions. The B value represents the δ^{15} N natural abundance of BNF derived N of nodulated *L. angustifolius* cultivated without N. In the present study, the B value of *L. angustifolius* was determined as -0.35.

The obtained %NDFA values were used to determine the proportion of N that originated from the atmosphere through fixation (N_{atm}), as well as the proportion of N that was taken up from the sand via the roots (N_{sand}). The efficiency of N_2 fixation (BNF efficiency) was also calculated by expressing the amount of fixed N relative to the N mass of the nodules (Kohl et al. 1983; Wanek and Arndt 2002). The effect of the different treatments on the mineral nutrition of *L. angustifolius* was investigated by calculating the specific N absorption and utilization rates. The specific N absorption rate (SNAR) was calculated using the equation provided by Nielsen et al. (2001) and represents the net N absorption rate per unit root DW (mmol N g^{-1} DW d^{-1}):

$$\text{SNAR} = \left(\frac{M_2 - M_1}{t_2 - t_1}\right) \times \left(\frac{\log_e R_2 - \log_e R_1}{R_2 - R_1}\right) \quad (3)$$

where *M* is the N content per plant, *t* is the time and *R* is the root DW.

In addition, Eq (3) was modified to calculate the N absorption rate from the atmosphere through fixation ($SNAR_{Atm}$) by substituting *M* with the amount of N_{atm} and *R* was substituted with the nodule DW. To calculate the rate of N absorption from sand ($SNAR_{sand}$), Eq (3) was modified by substituting *M* with the amount of N_{sand}. The specific N utilization rate (SNUR), which is a measure of the DW gained for the amount of N taken up by the plant (g DW $mmol^{-1}$ N d^{-1}) (Nielsen et al. 2001), was calculated using the following equation:

$$\mathrm{SNUR} = \left(\frac{w_2 - w_1}{t_2 - t_1}\right) \times \left(\frac{\log_e M_2 - \log_e M_1}{M_2 - M_1}\right) \quad (4)$$

where *M* represents the N content of the plant and *W* is the plant DW.

Phosphorous content of the milled *L. angustifolius* plants was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). Of the milled plant samples, *c.* 0.002 g of each sample was microwave digested (MARS Xpress, CEM corporation, Matthews, NC, USA) in 7 mL HNO_3. The microwave program was set at 100% power level (1600 W) with a ramp time of 25 min, pressure at 800 psi, temperature of 180 ˚C and a hold time of 10 min. The samples were allowed to cool in the digestion vessels and were subsequently transferred to 50 mL centrifuge tubes containing 43 g de-ionized water. Digestion blanks and NIST peach leaf standards were included as controls. A Thermo ICap 6200 ICP-AES (Thermo Scientific) was used to perform the analysis. Calibration standards were obtained by diluting certified standards (Merck) with Sigma Trace Select HNO_3 (Sigma-Aldrich) and distilled water. A separate NIST traceable standard was used to create calibration verification standards and after every 12 samples a drift check standard was analyzed.

2.5. IAA production and ACC deaminase activity

To determine whether *P. laurentii* CAB 91 could produce IAA *in vitro*, the yeast was cultivated overnight in 30 mL YM broth, contained in a 250 mL conical flask, at 26 ˚C on an Excella E10 platform shaker (100 rpm). The cells were pelleted by centrifugation and washed twice with sterile PSS as described earlier. The concentration of the resulting yeast suspension was set at log 6 cells mL^{-1}, from which 100 µL was used to inoculate test tubes containing either 10 mL Dworkin and Foster (DF) minimal medium (control) (Dworkin and Foster 1958; Penrose and Glick 2003) or 10 mL DF minimal

medium containing 0.1% (w/v) tryptophan (DF_{trp}; Sigma-Aldrich), in triplicate. The tubes were incubated on a TC-7 tissue culture roller drum (60 rpm) at 26 ˚C for 4 d, and the IAA production in both the control and DF_{trp} tubes were quantified daily. For this purpose, 1.2 mL of each culture was taken from the tubes and centrifuged (12000 **g**; 5 min; 4 ˚C). Thereafter 1 mL of the resulting supernatant was mixed with 2 mL of $FeCl_3$-$HClO_4$ reagent (Gordon and Weber 1951). The mixtures were incubated for 25 min in the dark, whereafter a SmartSpec Plus spectrophotometer (BioRad Laboratories Ltd., Johannesburg, South Africa) was used to quantify IAA production at a wavelength of 530 nm. To calculate the amount of IAA produced, a calibration curve was prepared by mixing a series of IAA concentrations, in triplicate, with the $FeCl_3$-$HClO_4$ reagent. The resulting mixtures were incubated for 25 min in the dark, after which the absorbances were measured at 530 nm.

The ability of *P. laurentii* CAB 91 to produce the enzyme ACC deaminase was determined by measuring the growth of the yeast on ACC as sole N source (Dell'Amico et al. 2005). Test tubes containing liquid DF minimal medium, devoid of a N source (DF_0) or with either $(NH_4)_2SO_4$ (DF_{NH}) or 0.3 *M* ACC (DF_{ACC}; Sigma-Aldrich) as sole N source, were prepared as described by Penrose and Glick (2003). A log 6 cells mL^{-1} suspension of *P. laurentii* CAB 91 was prepared as described earlier, of which 100 µL was inoculated into each tube in triplicate. The tubes were incubated for 7 d at 26 ˚C on a TC-7 tissue culture roller drum (60 rpm). The initial yeast concentration in the tubes, as well as growth, was enumerated daily by serially diluting 1 mL of each culture and spread plating the resulting dilutions onto YM_c agar plates. Colonies were counted after 2 d of incubation at 26 ˚C. In addition, the ACC deaminase activity of *P. laurentii* CAB 91 was determined by measuring the amount of α-ketobutyrate that is produced when the enzyme cleaves ACC (Honma and Shimomura 1978). This was achieved by using a modification of the colorimetric assay of Penrose and Glick (2003). For the induction of ACC deaminase, *P. laurentii* CAB 91 was cultured overnight at 26 ˚C in 30 mL YM broth contained in 250 mL conical flasks on an Excella E10 platform shaker (100 rpm), after which the cells were pelleted by centrifugation (3800 **g**; 5 min; 4 ˚C) and washed with 5 mL DF_0. Following an additional centrifugation step (3800 **g**; 5 min; 4 ˚C), the cells were resuspended in 8 mL DF_{NH} (control) or DF_{ACC}, in triplicate, and incubated for 14 h at 26 ˚C on a TC-7 tissue culture roller drum (60 rpm). Yeast protein extracts were subsequently prepared as described by Silve et al. (1992) with

modifications. The cells were harvested with centrifugation (3100 **g;** 7 min; 4 ˚C) and washed once with extraction buffer [0.2 *M* Tris-HCl (pH 8), 0.4 *M* $(NH_4)_2SO_4$, 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, 10% (v/v) glycerol, 0.001 *M* phenylmethylsulfonyl fluoride]. Following centrifugation at 3500 **g** for 10 min at 4 ˚C, the cell pellets were resuspended in 0.4 mL cold extraction buffer and transferred to chilled 2 mL microcentrifuge tubes that contained 0.3 mL glass beads (425 – 600 µm; Sigma-Aldrich). The cells were subsequently disrupted by vigorous mixing with a Vortex Genie 2 (Scientific Industries Inc., NY, USA) for a total of 6 cycles, each consisting of mixing for 30 s followed by 1 min of cooling on ice. The resulting suspensions were centrifuged at 13 000 **g** for 15 min at 4 ˚C and the protein-containing supernatants were collected. A 0.1 mL aliquot of each supernatant was stored at -20 ˚C for a Bradford protein quantitation (Bradford 1976), which was conducted using the BioRad protein assay kit II (BioRad Laboratories Ltd.). The remainder of these protein-containing supernatants was immediately assayed for ACC deaminase activity. Of each protein extract, 0.2 mL was placed in a glass tube and 0.02 mL of 0.5 *M* ACC was added to those extracts originating from the DF_{ACC} cultures (Penrose and Glick 2003). After thorough mixing, all tubes containing extracts from DF_{NH} and DF_{ACC} cultures were capped and incubated for 15 min at 30 ˚C. Subsequently, 0.8 mL of 0.56 *M* HCl was added to each tube, followed by 0.3 mL of 2, 4 dinitrophenylhydrazine (DPH; Merck) reagent (0.2% (w/v) DPH in 2 *M* HCl) and the contents were mixed. The tubes were incubated for 30 min at 30 ˚C after which 2 mL of 2 *M* NaOH was added to each tube. The contents were subsequently vortexed and the absorbance was measured at 540 nm with a SmartSpec plus spectrophotometer. Extracts from the DF_{NH} cultures were used to determine the contribution of the extract and reagents to the absorbance at 540 nm. In addition, the absorbance of the reagents together with ACC was also determined at 540 nm. These values together with an α-ketobutyrate standard curve, ranging from 0 – 1.0 µmol, were used to calculate the amount of α-ketobutyrate mg^{-1} protein h^{-1} that was produced in each tube (Penrose and Glick 2003).

2.6. Statistical analyses

Data are presented as mean ± 1SE. Normality of the data was checked using a Shapiro-Wilk's test. Although data was found to be non-normal, analysis of variance (ANOVA) was used on all data sets, to assess the effects and interactions of the treatments (Statistica v. 11, Statsoft, Tulsa, OK, USA), since ANOVA is known to be

robust against the violation of the assumption of a normal distribution (Schmider et al. 2010). Means where compared at $P < 0.05$ level using a *post hoc* Fisher's LSD multiple range test.

3. Results

3.1. Isolation and identification of yeasts from the rhizosphere of *L. angustifolius*

A total of 21 isolates were randomly selected from the YM_c agar plates, which originated from the yeast enrichment of *L. angustifolius* roots. These isolates were classified into two groups (A and B) by means of RFLP analysis of the ITS region. One isolate (5%) was assigned to group A, while the remaining 20 isolates (95%) were allocated to group B. The isolate representing group A was identified as *Cryptococcus friedmannii* (CAB 90; GenBank accession number KP789311), while an isolate representative of group B was identified as *Papiliotrema laurentii* (syn. *Cryptococcus laurentii*) (CAB 91; GenBank accession number KP789312).

3.2. Biomass parameters, nodules and growth respiration

The *L. angustifolius* plants treated with CLMF and CL were found to have a greater root, shoot and whole-plant biomass (Fig. 2.1a), as well as greater root, shoot and whole-plant RGRs (Fig. 2.1b), than control and MF treated plants. No significant differences in root, shoot and whole-plant biomass, as well as RGRs, were observed between CLMF and CL treated plants or between control and MF treated plants.

Plants treated with CL had the lowest nodule biomass and nodule RGR (Fig. 2.1c) compared to the other three treatments. Nodule biomass of plants treated with CLMF was greater than that of MF treated plants, yet the nodule biomass of both treatments did not differ significantly from that of control plants. Plants treated with CLMF had the greatest nodule RGR, whereas no significant difference was observed between control plants and those treated with MF. The %nodules per symbiotic root of CLMF, CL and MF treated plants were significantly lower than that of control plants (Fig. 2.1d). Additionally, the %nodules per symbiotic root of MF treated plants were greater than that of CL treated plants. The greatest BGA was observed for plants treated with either CLMF or CL, indicating that these plants invested more in root development than control and MF treated plants (Fig. 2.1e). Yet, no significant difference in BGA was

detected between CLMF and CL treated plants, as well as between control and MF treated plants.

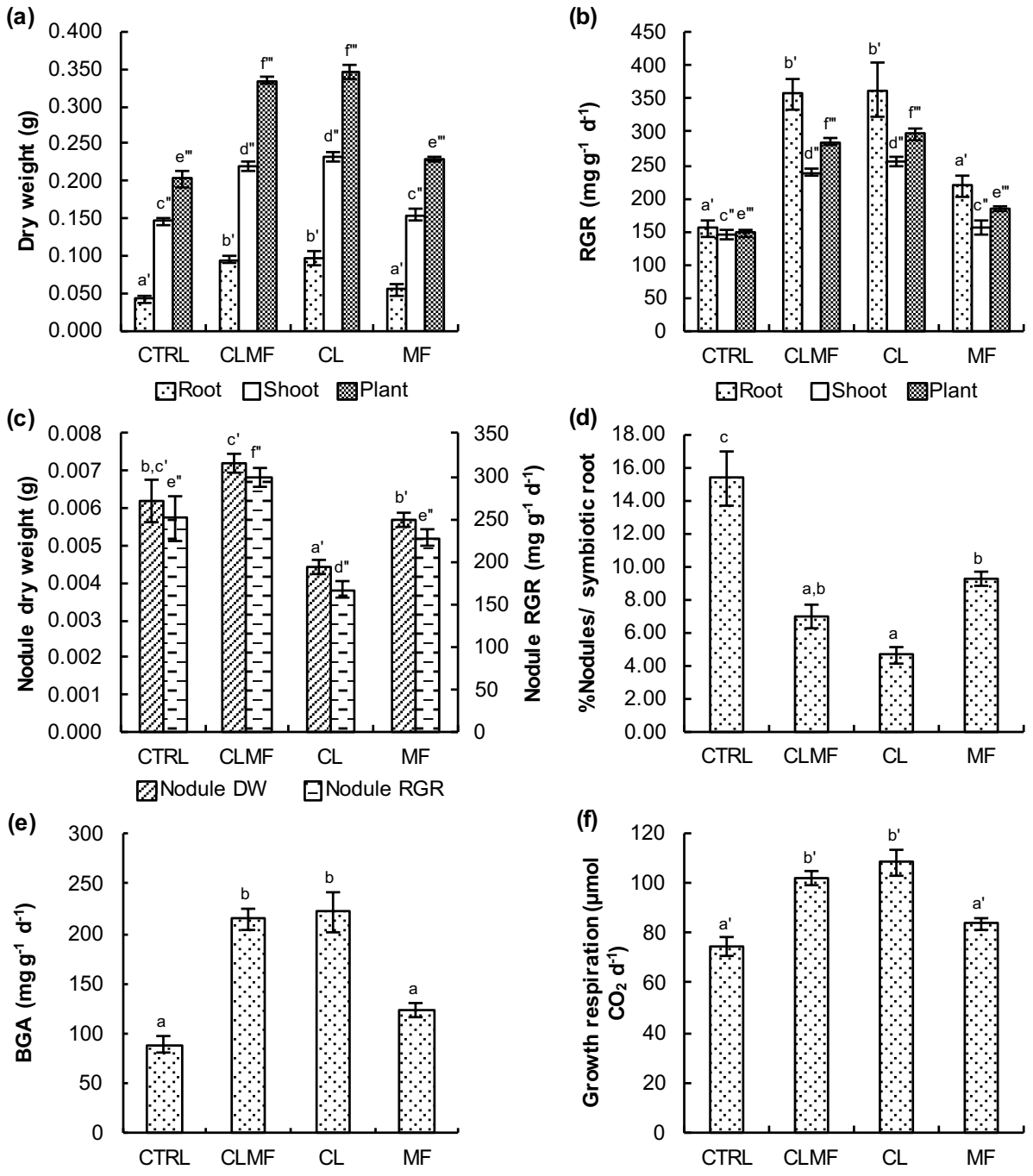

Fig. 2.1 Above- and below-ground growth parameters of *Lupinus angustifolius* L. in the presence of nodulating bacteria (Ctrl); or nodulating bacteria, *Papiliotrema laurentii* CAB 91 and mycorrhizal fungi (CLMF); or nodulating bacteria and *P. laurentii* CAB 91 (CL); or nodulating bacteria and mycorrhizal fungi (MF). After cultivation in a low phosphorus environment (10 µ*M*) for 4 weeks, plants were analyzed for root, shoot and whole-plant biomass (dry weight [DW]) **(a)**, root, shoot and whole-plant relative growth rate (RGR) **(b)**;

nodule biomass and RGR **(c)**, percentage nodules per symbiotic root **(d)**, total below ground allocation (BGA) **(e)** and growth respiration **(f)**. Bars denote the means of each treatment (n = 5) and error bars represent 1SE. The different letters indicate significant differences between the treatments ($P < 0.05$) and prime lettering indicates comparisons within the same parameter.

Concurrent with the root and shoot biomass of CLMF and CL treated plants, the growth respiration of these plants was enhanced in comparison to control plants and those treated with MF (Fig. 2.1f). No difference in growth respiration could be observed between control and MF treated plants, or between CL and CLMF treated plants.

3.3. Symbiont colonization and %NDFA

Mycorrhizal colonization (Fig. 2.2a) of CLMF treated plants was significantly greater than that of plants treated with MF. In contrast, colonization of the rhizosphere by *P. laurentii* CAB 91 (Fig. 2.2b) was less for CLMF treated plants (log 4.43 ± 0.068 cells g^{-1} root DW) compared to those treated with CL (log 4.89 ± 0.109 cells g^{-1} root DW). However, the yeast numbers present in the rhizosphere of both CL and CLMF treated plants decreased from the initial concentration of log 5.68 ± 0.23 cells g^{-1} root DW, which was measured directly after seedling roots were treated with the *P. laurentii* CAB 91 suspension.

The %NDFA did not differ significantly between control, CLMF, and MF treated plants (Fig. 2.2c). However, plants treated with CL had a greater %NDFA than plants treated with either CLMF or MF, but did not differ from control plants. In addition, these CL treated plants demonstrated the greatest BNF efficiency (Fig. 2.2d), while plants treated with CLMF had the lowest BNF efficiency. Furthermore, no difference in BNF efficiency was observed between control and MF treated plants.

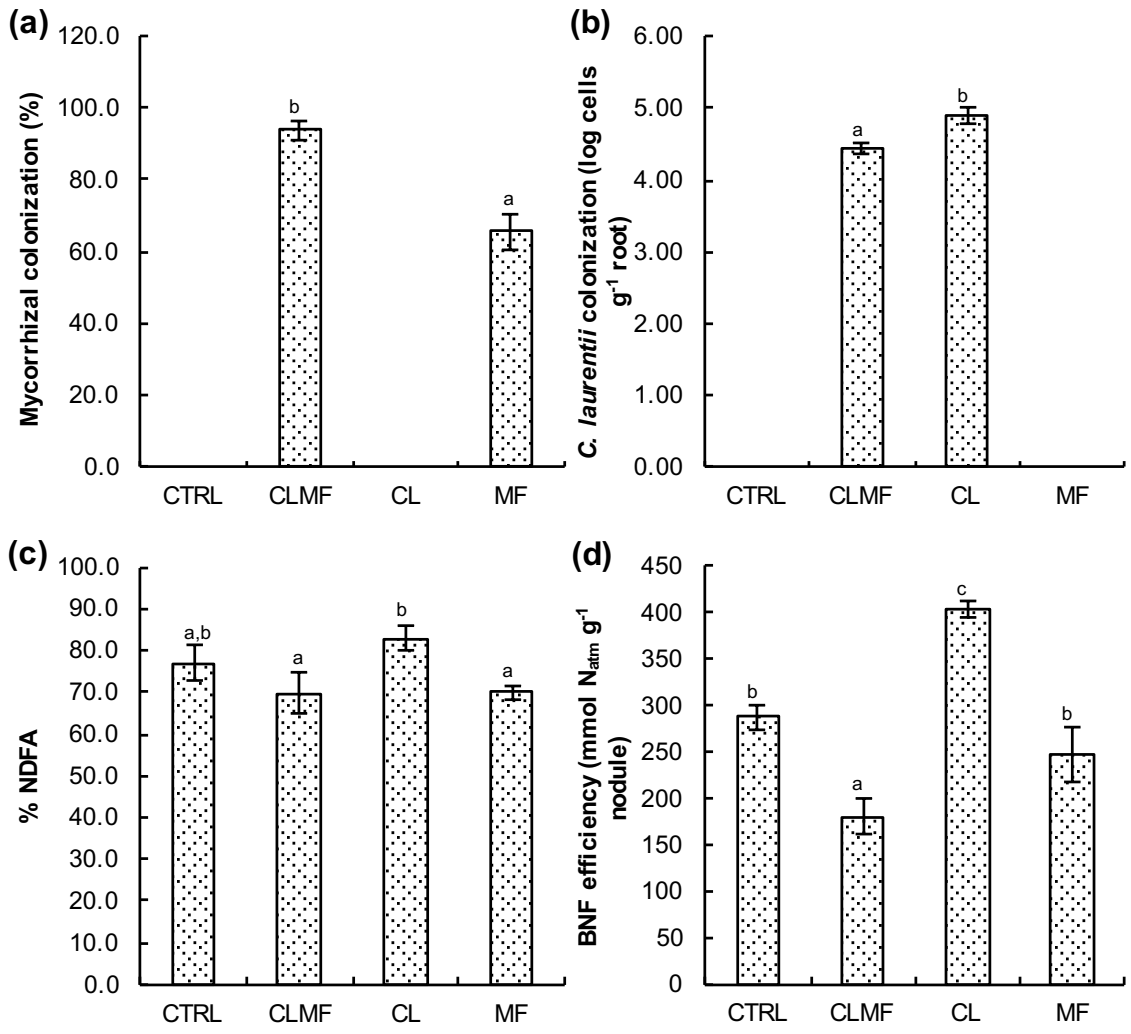

Fig. 2.2 Symbiotic parameters of *Lupinus angustifolius* L., cultivated in a low phosphorus environment (10 µ*M*) for 4 weeks, in the presence of nodulating bacteria (Ctrl); or nodulating bacteria, *Papiliotrema laurentii* CAB 91 and mycorrhizal fungi (CLMF); or nodulating bacteria and *P. laurentii* CAB 91 (CL); or nodulating bacteria and mycorrhizal fungi (MF). After harvesting the mycorrhizal colonization **(a)**; colonization of the rhizosphere by *P. laurentii* CAB 91 **(b)**; percentage of nitrogen derived from the atmosphere (%NDFA) **(c)** and biological nitrogen fixation (BNF) efficiency **(d)** were determined. Treatment means are given by the bars (n = 5) and the error bars denote 1SE. The different letters indicate significant differences between the treatments ($P < 0.05$).

3.4. Mineral nutrition

The total N concentration of CL treated plants was greater than that of plants treated with CLMF and MF (Fig. 3a). Yet, the N concentration of CL treated plants did not

differ significantly from control plants. The highest concentration of N_{atm} was observed for plants treated with CL, whereas no difference in N_{atm} was observed between control, CLMF treated or MF treated plants (Fig. 3a). When the concentration of N_{sand} was calculated for the four treatments, no significant difference was detected between control, CLMF, CL and MF treated plants (0.458 ± 0.082, 0.563 ± 0.085, 0.413 ± 0.090, 0.557 ± 0.023 mmol N g^{-1}, respectively; $P > 0.05$).

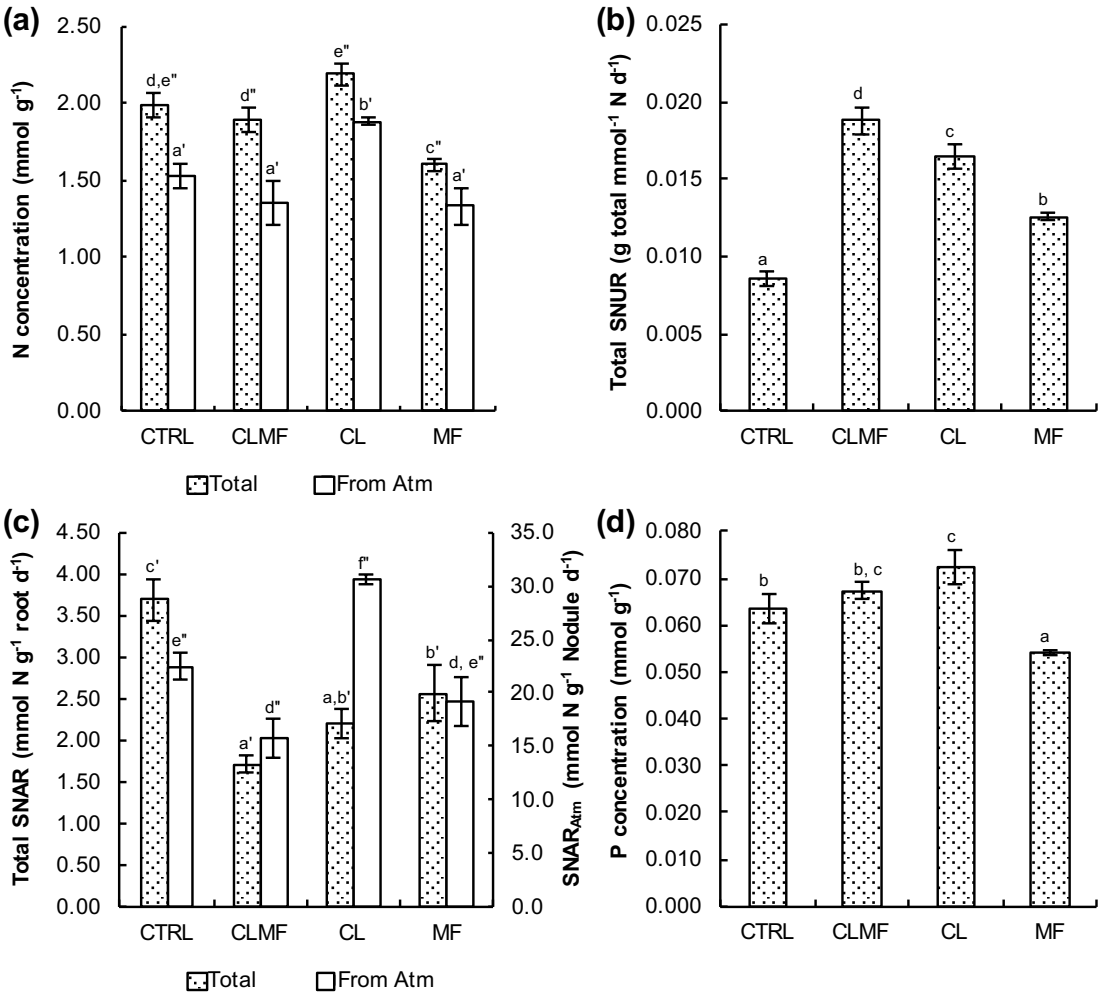

Fig. 2.3 Mineral nutrition of *Lupinus angustifolius* L. that was cultivated in the presence of nodulating bacteria (Ctrl); or nodulating bacteria, *Papiliotrema laurentii* CAB 91 and mycorrhizal fungi (CLMF); or nodulating bacteria and *P. laurentii* CAB 91 (CL); or nodulating bacteria and mycorrhizal fungi (MF) for 4 weeks in a low phosphorus (P) environment (10 µ*M*). Each treatment was analyzed for total nitrogen (N) concentration and nitrogen originating from the atmosphere (Atm) **(a)**; total specific N utilization rate (SNUR) **(b)**; total specific N absorption rate (SNAR) and SNAR from atmosphere ($SNAR_{atm}$) **(c)**, as well as P concentration **(d)**. The bars represent treatment means (n = 5), while the error bars denote 1SE. Different

lettering designates significant differences between the treatments ($P < 0.05$) and prime lettering indicates comparisons within the same parameter.

Plants treated with CLMF had the greatest SNUR, followed by those treated with CL (Fig. 3b). Plants treated with MF had the third highest SNUR, while control plants had the lowest SNUR. In contrast, control plants had the greatest total SNAR, while CLMF and CL treated plants had the lowest SNAR (Fig. 3c). In addition, no difference in total SNAR was observed between the latter two treatments. Similarly, MF treated plants did not differ significantly from plants treated with CL with regards to total SNAR. The greatest $SNAR_{atm}$ was observed for CL treated plants (Fig. 3c), while those treated with CLMF and MF did not differ from each other with regards to $SNAR_{atm}$. In addition, there was no significant difference in $SNAR_{atm}$ between plants treated with MF and control plants. Concurrent with the N_{sand} results, there was no significant difference in the $SNAR_{sand}$ between control, CLMF, CL and MF treated plants (0.635 ± 0.144, 0.429 ± 0.098, 0.468 ± 0.182, and 0.634 ± 0.034 mmol N g^{-1} root DW d^{-1}, respectively; $P > 0.05$).

When the P concentration was determined, it was observed that plants treated with MF had the lowest P concentration in their tissue (Fig. 3d). The P concentration in CL treated plants was greater than that of control plants, but no significant difference in P concentration was observed between plants treated with CL or CLMF. Similarly, CLMF treated plants did not differ from control plants with regards to P concentration.

3.5. IAA production and ACC deaminase activity

The highest IAA production (25.03 ± 1.70 µg mL^{-1}) by *P. laurentii* CAB 91 was observed on the fourth day of growth in the DF_{trp} medium. When the ability of this yeast to grow on ACC as sole N source was tested, no growth was observed in the DF_0 tubes. In contrast, growth was observed in the DF_{NH} and DF_{ACC} tubes, since the initial cell concentration increased from log 3.42 ± 0.01 cells mL^{-1} to log 4.81 ± 0.10 cells mL^{-1} and from log 3.40 ± 0.10 cells mL^{-1} to log 4.65 ± 0.05 cells mL^{-1}, respectively. Subsequent colorimetric analysis of ACC deaminase activity within protein extracts of *P. laurentii* CAB 91 revealed an activity of 318.31 nmol α-ketobutyrate mg^{-1} protein h^{-1}.

4. Discussion

It is well known that yeasts may occur in the rhizosphere (Botha 2006). Therefore, it was not surprising that representatives of *C. friedmannii* and *P. laurentii* were isolated from the rhizosphere of free-growing blue lupin during this study. Since some *P. laurentii* strains are able to improve growth of leguminous (Sampedro et al. 2004; Boby et al. 2008) and non-leguminous (Cloete et al. 2009; Cloete et al. 2010) plants, it seemed likely that *P. laurentii* CAB 91 may act as a PGPY of blue lupin. Therefore, this yeast strain was used during experimentation in the present study and assessed for two plant growth promoting traits. Colorimetric detection of IAA production using Salkowski reagent, revealed that *P. laurentii* CAB 91 could produce IAA equivalent to *ca.* 25 µg/ mL. Although this reagent is used by various research groups for the detection of IAA production by yeasts (Nassar et al. 2005; Xin et al. 2009; Amprayn et al. 2012; Deng et al. 2012; Nutaratat et al. 2014), it should be noted that other indolic compounds (Glickmann and Dessaux 1995), particularly indolepyruvic acid, are detected with this reagent. Therefore, it is imperative that the IAA production by *P. laurentii* CAB 91 is analyzed with high performance liquid chromatography (HPLC) in the future. Nevertheless, the IAA concentration produced by this yeast was similar to (Nassar et al. 2005; El-Tarabily 2008) or higher than (Amprayn et al. 2012; Deng et al. 2012) that detected for other PGPY, when the same reagent was used. Analysis for the ability of *P. laurentii* CAB 91 to deaminate ACC, revealed that this yeast not only utilized ACC as sole N source, but also showed an ACC deaminase activity of 318.31 nmol α-ketobutyrate mg^{-1} protein h^{-1}. Considering that an ACC deaminase activity of 300 nmol α-ketobutyrate mg^{-1} protein h^{-1} or more is accepted as high (Penrose and Glick 2003), it is evident that *P. laurentii* CAB 91 is a good producer of this enzyme. To date, ACC deaminase activity has been quantified for only one other PGPY, *i.e. Cryptococcus* sp., which was recorded to have an activity of 0.06 ± 0.01 µ*M* α-ketobutyrate mg^{-1} protein h^{-1} (Deng et al. 2012). Taken together, our results indicate that *P. laurentii* CAB 91 possess plant growth promoting characteristics.

Our results indicated that growth and nodulation of blue lupin were unaffected when treated with mycorrhizal fungi, even though plants were notably colonized. This colonization is unusual, since the genus *Lupinus* is considered to be non-mycorrhizal (Jones 1924; Avio et al. 1990; Vierheilig et al. 1994; Oba et al. 2001, 2002). However,

mycorrhizal colonization is known to occur in some lupins (Trinick 1977; Allen et al. 1984; O'Dell and Trappe 1992), but the plants do not benefit nutritionally from the association (Lambers and Teste 2013). Similarly, the lower N and P concentrations observed in the present study for MF treated plants indicate that *L. angustifolius* gained no nutritional benefit, while allocating some nutritional resources to the mycorrhizal symbionts. The unusual mycorrhizal colonization witnessed for these plants might be attributed to the presence of a variety of mycorrhizal fungi in the commercial inoculum used during this study, since the usage of different mycorrhizal fungi as inocula were limited in previous studies wherein the mycorrhizal status of *L. angustifolius* was evaluated (Jones 1924; Thomas 1943; Thompson and Wildermuth 1989; Avio et al. 1990; Oba et al 2001, 2002). In addition, evidence for the ability or inability of *C. etunicatum*, *G. gigantea, R. clarus* and *P. occultum* to colonize *L. angustifolius* roots is lacking. Considering that the commercial inoculum used in the present study was not sterile, it is also possible that helper bacteria was introduced into the pots. These bacteria are known to stimulate mycorrhizal colonization (Meyer and Linderman 1986; Requena et al. 1997; Valdenegro et al. 2001; Khan and Zaidi 2007), which might have affected mycorrhizal colonization of *L. angustifolius.*

It is known that yeasts may stimulate growth of nodulated legumes that are either colonized (Singh et al. 1991; Medina et al. 2004a; Medina et al. 2004b; Azcon et al. 2010; Morsy 2015) or uncolonized (Tuladhar and Subba Rao 1985; Morsy et al. 2014) by mycorrhizal fungi. Similarly, during the present study we found that biomass of blue lupin was increased when *P. laurentii* CAB 91 was present in the rhizosphere of CL and CLMF treated plants. This increase was underpinned by the elevated growth respiration, as well as root and shoot RGRs, of these lupins. In addition, the high root RGR and increased BGA observed for these plants indicate that *P. laurentii* CAB 91 stimulated root growth of blue lupin. This concurs with the work of Cloete et al. (2009) where it was found that another *P. laurentii* strain could improve root growth of buchu, which was attributed to the ability of the yeast to act as a nutrient scavenging symbiont of its host (Cloete et al. 2010). It was speculated that this yeast increased the plant's P concentration by accumulating short chain inorganic polyphosphates. Thus, the improved P status observed in the present study for CL and CLMF treated plants in comparison to control plants, might be attributed to *P. laurentii* CAB 91 supplying the plant with P. Not surprisingly, the P concentrations of all plants cultivated in this study

(Fig. 3d), were lower than that of *L. angustifolius* (151.7 µmol P g^{-1}) cultivated in the presence of sufficient P (Hocking and Pate 1978), since our plants were provided with a low amount of P (10 µM). The improved P nutrition of CL and CLMF plants was, however, not the sole contributor to increased plant growth, as an altered N nutrition also influenced the growth of these plants.

Considering that the N concentration of all *L. angustifolius* plants ranged between 1.6 and 1.98 mmol N g^{-1} (Fig. 3a), it seems that the plants contained similar amounts of N than *L. angustifolius* cultivated under sufficient nutrient supply (1.49 mmol N g^{-1}) (Hocking and Pate 1978). Yet, in the present study we found that blue lupin treated with CL and CLMF followed two different N growth strategies depending on the presence of mycorrhizal fungi. Treating the plants with *P. laurentii* CAB 91 alone resulted in reduced nodule biomass. Yet, these plants maintained a total N concentration similar to that of control plants, most likely through increased N_2 fixation, since BNF efficiency, N_{atm} concentration and N_{atm} uptake rate of CL treated plants were increased. Therefore, it seems that *P. laurentii* CAB 91 had a positive effect on the BNF of blue lupin. To the best of our knowledge, this is the first report on the ability of a rhizosphere yeast to influence the BNF of its host plant. This phenomenon was, however, demonstrated for PGPR (Dashti et al. 1998; Chebotar et al. 2001; Valverde et al. 2006; Figueiredo et al. 2008; Yadegari et al. 2010) and some mechanisms whereby these bacteria affect BNF were described previously. For instance, the production of IAA and ACC deaminase by PGPR is known to increase nodule ATP and longevity respectively (Takahashi et al. 2012; Gontia-Mishra et al. 2014). Considering that during the present study we found that *P. laurentii* CAB 91 was able to produce IAA and ACC deaminase *in vitro,* it seems possible that this yeast influenced the BNF of CL treated plants in a similar manner to PGPR.

In the presence of both *P. laurentii* CAB 19 and mycorrhizal fungi the N growth strategy of blue lupin entailed a greater reliance on nodule RGR and efficient growth on available N resources (SNUR), since BNF efficiency and $SNAR_{atm}$ of these plants were reduced. Considering that nodule biomass of CL treated plants was less than that of CLMF treated plants it seems that mycorrhizal fungi was responsible for increased nodule RGR. Similarly, it was demonstrated that legume nodulation is increased by mycorrhizal fungi (Smith et al. 1979; Barea and Azcon-Aguilar 1983; Pacovsky et al.

1986). In addition, it was shown that yeasts can improve AMF colonization of legumes (Singh et al. 1991; Sampedro et al. 2004; Boby et al. 2007, 2008) and our results indicated that *P. laurentii* CAB 91 improved mycorrhizal colonization of blue lupin under glasshouse conditions. By doing so, this yeast impacted the N nutrition and growth of CLMF treated plants. This finding might hold practical implications for the cultivation of blue lupin in N-limited soil.

In conclusion, this study provides evidence that the rhizosphere yeast *P. laurentii* CAB 91 could form tripartite (without mycorrhizal fungi) and quadripartite (with mycorrhizal fungi) symbioses with nodulated blue lupin, which resulted in improved plant growth. In addition, the type of N growth strategy employed by blue lupin was contingent upon whether the symbiosis was tripartite or quadripartite. From these results, it seems likely that the nutritional physiology of blue lupin can be enhanced by utilizing yeasts as bio-fertilizers. Our results are, however, preliminary, since conditions used in this study differ considerably from that in the field. Nevertheless, pot trials are a valuable tool for the initial study of plant interactions, as variability and experimental error is reduced (Knight and Will 1971). Furthermore, when differences are not detected under controlled conditions in pot trials, it is likely that under more variable field conditions differences will not be detected either. Yet, in order to confirm whether yeasts may be used as bio-fertilizers to improve the performance of *L. angustifolius* in low nutrient soils, field studies are needed. Furthermore, the mycorrhizal status of *Lupinus* needs to be investigated, preferably using different mycorrhizal fungi in the presence of helper bacteria. Lastly, the mechanisms at play when *P. laurentii* CAB 91 is present in either a tripartite or quadripartite symbiosis with blue lupin needs to be evaluated. Such information will improve our chances for selecting the most beneficial combination of symbionts to improve lupin growth and nutrition.

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Chapter 3 – The yeast *Papiliotrema laurentii*: a potential bio-fertiliser for use in crop rotation

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1. Introduction

Global use of nitrogen (N) fertilisers in agriculture is extensive and a large quantity of N input can be attributed to cultivation of *Triticum aestivum* L. (wheat) (Diacono et al., 2013). Yet, over application of N fertilisers decreases wheat yield (Kong et al., 2017) and has a negative impact on the environment (Adesemoye and Kloepper, 2009; Smil, 2000). This has led to the employment of alternative measures to increase N levels in soil, such as utilisation of N-fixing legumes in break-crop and crop rotation systems (Costanzo and Bàrberi, 2014). Since leguminous plants can be cultivated in nutrient depleted soils (Ma et al., 1997; Maroko et al., 1999; Sanchez, 1999), they are often used in sustainable agriculture to fix dinitrogen (N_2). One such a legume is *Lupinus angustifolius* L. (blue lupin), which is used in break-crop and crop rotation systems to increase cereal yields in Mediterranean climates (McNeill and Fillery, 2008; Wijayanto et al., 2009) and wheat yields in Australia (Angus et al., 2015; Hane et al., 2017). In addition to the usage of blue lupin in break-crop and crop rotation practices; this plant is now considered to be a human health food (Hane et al., 2017; Stephany et al., 2016). Due to the threat of climate change on food security (FAO, 2016) together with the need to sustainably increase food production to meet the demands of a growing population, it is imperative that production of both blue lupin and wheat is increased beyond that of current management technologies.

One manner wherein plant growth can be increased is the usage of plant growth promoting yeasts (PGPY). These unicellular fungi are known to improve growth of various plants (Amprayn et al., 2012; Azcon et al., 2010; Cloete et al., 2009; Deng et al., 2012; Fu et al., 2016; Liu et al., 2016; Moller et al., 2016) via direct and indirect mechanisms. Indirect mechanisms of plant growth promotion by PGPY include inhibition of phytopathogens (Fu et al., 2016; Silambarasan and Vangnai, 2017; Wang et al., 2013) and stimulation of mycorrhizal fungi (Alonso et al., 2008; Fracchia et al., 2003; Gollner et al., 2006; Sampedro et al., 2004; Souchie et al., 2010). Direct mechanisms include production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Amprayn et al., 2012; Deng et al., 2012; Liu et al., 2016; Moller et al., 2016; Wang et al., 2013), indole-3-acetic acid (IAA) (Nassar et al. 2005; Mukherjee and Sen 2014; Moller et al. 2016; Silambarasan and Vangnai 2017), and polyamines (PAs) (Amprayn et al., 2012; Cloete et al., 2009), as well as the solubilisation of phosphate (Nakayan et al., 2013; New et al., 2013; Vassilev et al., 2001) and zinc (Fu

et al., 2016). Additionally, it was demonstrated that the PGPY *Papiliotrema laurentii* (syn. *Cryptococcus laurentii*) CAB 578, originating from the rhizosphere of the schlerophyllous shrub, *Agathosma betulina* (Berg.) Pillans) (buchu), is able to increase the photosynthetic rate and photosynthetic water use efficiency (PWUE) of this medicinal plant (Cloete et al., 2010b), thereby increasing plant growth.

In addition to positive effects on plant growth, yeasts can increase the germination of a wide range of plants (Akhtyamova and Sattarova, 2013; Gaballah and Gomaa, 2004; Matić et al., 2014; Nakayan et al., 2013; New et al., 2013; Ramos-Garza et al., 2016; Shalaby and El-Nady, 2008). Although increased germination can result in greater crop yield (Finch-Savage and Bassel, 2016), germination data might not predict seedling emergence under field conditions (Finch-Savage and Bassel, 2016; Marcos Filho, 2015), since germination experiments only provide information on the ability of seeds to germinate under controlled laboratory conditions (Finch-Savage and Bassel, 2016; Hampton and Coolbear, 1990; Hampton and TeKrony, 1995). It was, however, suggested that seeds exhibiting high vigour under controlled laboratory conditions may perform well in the field (ISTA, 2014). Seed vigour is defined as 'the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments; a vigorous seed lot is one that is potentially able to perform well under environmental conditions which are not optimal for the species'. Therefore, if PGPY are capable of improving germination, it is likely that these yeasts may also increase seed vigour. However, to date no study has been aimed at testing this hypothesis.

Moreover, the role of a single yeast strain in the germination and growth of plants used together within a break-crop or crop rotation system has largely remained unexplored. Based on host-*P. laurentii* interactions that resulted in improved growth and N nutrition of blue lupin (Moller et al., 2016; Chapter 2), as well as increased growth, phosphorous (P) nutrition and photosynthesis of buchu (Cloete et al., 2010a; Cloete et al., 2010b), it was hypothesised that this yeast could promote the germination and developmental physiology of both blue lupin and wheat. To test this hypothesis, we compared the plant growth promoting traits of *P. laurentii* CAB 91 to that of two other rhizosphere yeasts, *i.e. Hannaella zeae* CAB 1119 and *Saitozyma podzolica* CAB 1199. In addition, we evaluated the influence of *P. laurentii* CAB 91 on germination and vigour

of blue lupin and wheat in comparison to that of *H. zeae* CAB 1119 and *S. podzolica* CAB 1199. Lastly, we investigated the interaction between each yeast strain and each plant in pot trials, by measuring plant growth, relative growth rates and photosynthetic parameters after one and two months of growth.

2. Materials and Methods

2.1. Yeast strains and maintenance

Yeasts originating from the rhizosphere of different plants (Table 3.1) were obtained from the culture collection at the Department of Microbiology, Stellenbosch University. Working cultures of each strain were maintained on yeast extract-malt extract (YM; Yarrow, 1998) agar slants at 23 ˚C. In addition, stock cultures of the strains were stored at -80 ˚C in 15% (v/v) glycerol.

Table 3.1. Yeast strains, their origins and accession numbers, used during this study.

Yeast strain	Origin	GenBank accession no.[a]	Reference
Hannaella zeae CAB 1119	*Themeda triandra* rhizosphere	KY826437	Moller (2012)
Papiliotrema laurentii CAB 91	*Lupinus angustifolius* rhizosphere	KP789312	Moller et al. (2016)
Saitozyma podzolica CAB 1199	*Eragrostis trichophora* rhizosphere	KY826436	Moller, pers. comm.[b]

[a] Genbank accession number for the D1/ D2 rDNA gene sequence of each strain
[b] Moller, personal communication; strains were isolated using classical plate culture techniques

2.2. Plant growth promoting traits of yeast strains

2.2.1. Quantitative detection of IAA production

The *in vitro* production of IAA by the yeast strains listed in Table 3.1 was determined as described by Moller et al. (2016), except for *P. laurentii* CAB 91, whereof the IAA production was previously determined under identical conditions (Moller et al., 2016; Chapter 2). In brief, a log 6 cells mL^{-1} suspension was prepared for each strain as detailed by Moller et al. (2016). Thereafter, 0.1 mL of each yeast suspension was used to inoculate triplicate test tubes containing either 10 mL Dworkin and Foster minimal medium (DF; Dworkin and Foster, 1958; Penrose and Glick, 2003) or 10 mL DF supplemented with 0.1% (w/v) tryptophan (DF_{trp}; Sigma-Aldrich, St Louis, MO, USA).

The inoculated tubes were incubated for 4 days at 26 ˚C on a tissue culture roller drum (TC-7, New Brunswick Scientific CO. Inc., Edison, NJ, USA) set at 60 revolutions per minute (rpm). Production of IAA in both the DF and DF_{trp} tubes was quantified daily using the Salkowski reagent ($FeCl_3$-$HClO_4$) (Gordon and Weber, 1951) as described by Moller et al. (2016). Using pure IAA as a standard together with the Salkowski reagent, IAA production was quantified at a wavelength of 530 nm with a SmartSpec Plus spectrophotometer (BioRad Laboratories Ltd., Johannesburg, South Africa). To normalise IAA production for each of the yeast strains, growth in DF and DF_{trp} tubes was measured daily using serial dilution spread plates prepared with YM agar.

2.2.2. Qualitative and quantitative assessment of ACC deaminase activity

The activity of ACC deaminase was assessed for all yeasts strains (Table 3.1) except for *P. laurentii* CAB 91, since the ACC deaminase activity of this yeast strain was determined previously under identical conditions (Moller et al., 2016; Chapter 2). The ability of the yeast strains to produce this enzyme was first established qualitatively, by testing for growth on ACC as sole N source (Dell'Amico et al., 2005) as described by Moller et al. (2016). Thereafter ACC deaminase activity was quantified for each yeast strain by measuring the amount of α-ketobutyrate produced when the enzyme cleaves ACC (Honma and Shimomura, 1978) using the method provided by Moller et al. (2016). After determining the quantity of α-ketobutyrate and protein produced by each yeast strain, ACC deaminase activity of the organisms was expressed as α-ketobutyrate mg^{-1} protein h^{-1} (Penrose and Glick, 2003).

2.2.3. Qualitative determination of siderophore production

Siderophore production by the yeast strains (Table 3.1) was determined using the overlay chrome azurol S (O-CAS) assay (Pérez-Miranda et al., 2007) with modifications. A log 6 cells mL^{-1} suspension of each yeast strain was prepared as described by Moller et al. (2016). In addition, a log 6 cells mL^{-1} suspension of *Pseudomonas aeruginosa* PA01, which served as the positive control, was prepared in the same manner except that tryptone soy broth (TSB; Merck) was used as the initial growth medium. An aliquot of each cell suspension (20 µL) was spotted onto King's B agar (King et al., 1954) plates in triplicate and the plates were incubated at 23 ˚C. After 2 days of incubation, *ca.* 10 mL of O-CAS agar that was prepared as described by Louden et al. (2011), was poured over each culture. Subsequently, the

plates were incubated for 7 days at 23 ˚C and during this time the plates were inspected daily for the formation of yellow to orange halos around each colony in the overlay medium.

2.2.4. Qualitative detection of polyamine production

The production of PAs through the decarboxylation of arginine, lysine or ornithine by the yeast strains (Table 3.1) was assessed on Long Ashton decarboxylase (LAD) agar plates (Cloete et al., 2009). For this purpose, the LAD agar was supplemented with either 2 g L^{-1} arginine (Sigma-Aldrich), 3.36 g L^{-1} lysine (Sigma-Aldrich) or 3.87 g L^{-1} ornithine monohydrochloride (Sigma-Aldrich). In addition, LAD agar plates devoid of amino acids were included as a negative control. The plates were inoculated with the different yeasts (Table 3.1) by streaking three day old cultures grown on YM agar onto the LAD agar plates, in triplicate. In addition, *P. laurentii* CAB 578 (Cloete et al., 2009) was streaked in triplicate onto the LAD agar plates as a positive control. The plates were incubated for 7 days at 23 ˚C and inspected daily for the formation of red halo's around the colonies, which was indicative of decarboxylation activity and thus the potential production of PAs (Cloete et al., 2009; Nassar et al., 2003).

2.2.5. Quantitative assessment of PO_4^{3-} solubilisation

Solubilisation of PO_4^{3-} by the yeast strains (Table 3.1) was assayed in the National Botanical Research Institute's phosphate growth medium (NBRIP; Nautiyal, 1999) and Pikovskaya's medium (PVK; Pikovskaya, 1948) using a modified method of Nautiyal (1999). In addition, to determine whether the yeast strains assimilated free PO_4^{3-} released during the solubilisation of $Ca_3(PO_4)_2$, 4.39 g KH_2PO_4 was used as substitute for $Ca_3(PO_4)_2$ in both NBRIP ($NBRIP_{MOD}$) and PVK (PVK_{MOD}). Prior to experimentation, all glassware was rinsed with hot dilute (1 *M*) HCl to remove residual PO_4^{3-} (APHA, 2012). A log 6 cells mL^{-1} suspension of each yeast strain was prepared according to Moller et al. (2016), whereafter 1 mL of each suspension was added in triplicate to 500 mL conical flasks containing either 50 mL NBRIP, 50 mL PVK, 50 mL $NBRIP_{MOD}$ or 50 mL PVK_{MOD}. Uninoculated flasks that contained NBRIP, $NBRIP_{MOD}$, PVK and PVK_{MOD} were included in the experimentation as negative controls. Following inoculation, the cultures were incubated for 1 week at 26 ˚C on an Excella E10 platform shaker (New Brunswick Scientific CO. Inc.) set to 170 rpm.

To determine the initial and final concentration (*i.e.* after the 1 week incubation period) of free PO_4^{3-} present in each growth medium the 4500-P:E ascorbic acid method (APHA, 2012) was used with modifications. For the assay, 20 mL of the growth medium was removed from each flask and centrifuged at 8000 **g** (4 ˚C) for 10 min. Thereafter each supernatant was filtered through a grade 4 qualitative filter paper (Whatman PLC, Buckinghamshire, United Kingdom) and collected in a glass tube. To remove any remaining yeast cells the supernatants were passed through a 0.2 µm Minisart NML Plus cellulose acetate syringe filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) into clean glass tubes. Subsequently, a 1 in 500 dilution of each supernatant originating from the $NBRIP_{MOD}$ and PVK_{MOD} cultures was made in distilled water (dH_2O), while supernatants originating from NBRIP and PVK cultures were diluted 1:10 and 1:50 with dH_2O. Thereafter 1.6 mL of a molybdenum-ascorbic acid reagent (1.25 *M* H_2SO_4 [Merck], 0.4105 m*M* $K(SbO)C_4H_4O_2.½H_2O$ [Merck], 4.8555 m*M* $(NH_4)_6Mo_7O_{24}.4H_2O$ [Merck] and 0.03 m*M* ascorbic acid [Merck]) was added to 10 mL of each diluted supernatant and the contents were mixed thoroughly. After 10 min of incubation at 24 ˚C, the absorbance of each mixture was read at 880 nm with a Spectroquant Pharo 300 (Merck) spectrophotometer. The quantity of PO_4^{3-} solubilised by each yeast strain was determined by preparing a standard curve ranging from 0.1 to 1.5 mg L^{-1} PO_4^{3-} using pre-dried KH_2PO_4 (Merck) as described by APHA (2012), while taking into account the concentration of free PO_4^{3-} in the initial medium, as well as in the $NBRIP_{MOD}$ and PVK_{MOD} media after the growth period. To normalize PO_4^{3-} solubilisation for each yeast strain, the yeast numbers present in each medium directly after inoculation, as well as after 1 week of incubation, was determined. This was achieved by serially diluting 1 mL of the growth medium from each flask and spread-plating the dilutions onto YM agar. Colonies present on the plates were counted after 2 days of incubation at 26 ˚C and solubilised PO_4^{3-} (mg L^{-1}) was divided by the cell counts to standardise the PO_4^{3-} of each strain.

2.2.6. Qualitative estimation of Zn solubilisation

The yeast strains listed in Table 3.1 were evaluated for Zn solubilisation on solid media as described by Farokh et al. (2011). In brief, a log 6 cells mL^{-1} inoculum of each yeast strain was prepared as described previously (Moller et al., 2016). Subsequently, 10 µL of each inoculum was spotted in triplicate onto ZnO agar (Indiragandhi et al., 2008). The plates were incubated for 1 week at 23 ˚C and were inspected daily for the

formation of clear zones around each colony.

2.3. Effect of yeasts on seed germination and vigour

2.3.1. Seed surface sterilisation and yeast inoculum preparation

To evaluate the effect of the yeast strains (Table 3.1) on the germination and vigour of both blue lupin and wheat, seeds of *L. angustifolius* cv. Gunyidi (n = 2100; Agricol, Cape Town, South Africa) and *T. aestivum* cv. SST047 (n = 2100; Overberg Agri, Mooreesburg depot, Mooreesburg, South Africa) were surface sterilised as described by Moller et al. (2016). Inoculums of the yeast strains (Table 3.1) were prepared by culturing each organism in 30 mL YM broth contained in 250 mL conical flasks for 2 days at 26 ˚C on an Excella E10 rotary shaker (100 rpm). Subsequently, the cells were harvested by centrifugation (3800 **g**; 5 min; 4 °C) and a log 8 cells mL^{-1} suspension of each yeast strain was prepared according to Moller et al. (2016).

2.3.2. Germination and seedling evaluation

To determine the effect of the yeast strains on blue lupin and wheat germination, surface sterilised blue lupin (n = 100 per treatment) and wheat (n = 100 per treatment) seeds were submerged for 2 min in each of the log 8 cells mL^{-1} yeast suspensions. In addition, surface sterilised blue lupin (n = 100) and wheat (n = 100) seeds were suspended in sterile physiological saline solution (PSS) to serve as the negative control. Subsequently, the yeast treated and control seeds were dried under sterile conditions in a biosafety cabinet (LabAire, Germiston, South Africa) for 2 h, whereafter they were planted onto Murashige and Skoog (MS; Murashige and Skoog, 1962) agar. Thereafter, all seeds were allowed to germinate for 1 week in the dark at 23 ˚C. Each day the plates were inspected and germination was evaluated according to the guidelines of ISTA (1999). From the germination data, the mean germination time (MGT) was calculated using the equation of Labouriau (1983). In addition, the germination data was used to calculate Maguire's Speed of Germination (MSG) according to the equation provided by Maguire (1962).

2.3.3. Vigour and seedling evaluation

The influence of the yeast strains (Table 3.1) on blue lupin and wheat vigour was investigated using the cold test method (Hampton and TeKrony, 1995). This method can be used to evaluate the effect of fungicides or microorganisms present in soil

(Marcos Filho, 2015) on seed emergence. In addition, by using this method high heat or chemical treatment is avoided, thereby minimising factors which may inhibit yeast growth. For the cold test, surface sterilised blue lupin (n = 200 per treatment) and wheat (n = 200 per treatment) seeds were submerged in the log 8 cells mL^{-1} yeast suspensions for 2 min. In addition, surface sterilised blue lupin (n = 200) and wheat (n = 200) seeds submerged in sterile PSS served as negative controls. All seeds were dried under sterile conditions for 2 h in a biosafety cabinet (LabAire). Two sheets of germination paper (0.29 m x 0.58 m; Sartorius Stedim Biotech GmbH), pre-saturated with 45 mL sterile dH_2O and equilibrated at 10 ˚C overnight, were layered on top of each other. Thereafter, seeds (n = 50) were placed onto the double layer of germination paper and covered with 200 mL sterile acid washed silica sand (grain size ranging from 250 to 355 µm; Consol Glass (Pty) Ltd., Stellenbosch, South Africa). Another germination paper (pre-saturated and equilibrated at 10 ˚C) was subsequently placed over the sand and the three sheets were lightly rolled together. To attain 200 seeds per treatment, four rolls with 50 seeds each were prepared for each of the yeast treatments, as well as the control. After placing the rolls in sterile plastic containers (35.5 cm (h) x 25 cm (l) x 18 cm (b)), the containers were covered with black plastic bags (Garbie, Astrapak, Gillits, South Africa) to create a dark environment. The containers were incubated in the dark at 10 ˚C for 1 week, where after the containers where moved to an incubator for germination in the dark at 25 ˚C for 5 days. Seedlings were evaluated using the same guidelines for the standard germination test as outlined by ISTA (1999).

2.4. Influence of yeast strains on growth and photosynthesis of blue lupin and wheat

2.4.1. Seed surface sterilisation, preparation of sterile silica sand and yeast inoculums

Prior to experimentation all growth media, seeds and solutions were sterilised to ensure that the yeast inoculums were dominant in the rhizosphere, while decreasing the risk of contamination by phytopathogens. Therefore, seeds of blue lupin (n = 100) and wheat (n = 100) were surface sterilised as previously described (Moller et al., 2016). In addition, silica sand (600 µm grain size; Agrimark, Stellenbosch, South Africa) was acid washed with 0.1 *M* HCl and sterilised according to Moller et al. (2016).

For the inoculation of blue lupin and wheat, a log 8 cells mL^{-1} suspension of each yeast strain (Table 3.1) was prepared as detailed by Moller et al. (2016).

2.4.2. Inoculation of blue lupin with yeast strains

Prior to inoculation of blue lupin with the yeast strains (Table 3.1), the surface sterilised seeds (n = 100) were allowed to imbibe sterile dH_2O for 16 h, whereafter the seeds were treated with an inoculum of *Bradyrhizobium* sp. according to the manufacturer's specifications (Stimuplant, Zwavelpoort, South Africa). Subsequently, the seeds were sowed in a seeding tray containing *ca.* 10 g of sterile acid-washed silica sand (one seed per well) and allowed to germinate for 4 d in a well-ventilated glasshouse with an average light/ dark relative humidity of 50/ 80% and a temperature of 23/ 15 °C, as well as a 12 h photoperiod with a photosynthetic photon flux density of 1000 - 1100 µmol m^2 s^{-1}. Thereafter, seedlings of uniform size (n = 52) were dislodged from the silica sand and the roots were treated with either sterile PSS (n = 13) or with one of the suspensions prepared of *H. zeae* CAB 1119 (n = 13), *P. laurentii* CAB 91 (n = 13) or *S. podzolica* CAB 1199 (n = 13). After treatment, the seedlings were planted in pots (n = 10 per treatment), which contained *ca.* 800 g of the sterile acid-washed silica sand. The remaining seedlings (n = 3 per treatment) were used to determine the number of yeasts present on the roots directly after treatment. This was achieved by rinsing the roots in sterile PSS and preparing serial dilutions of the resulting suspensions. Thereafter the dilutions were spread plated onto YM agar and following 2 d of incubation at 26 ˚C, colonies were enumerated on the plates. After drying the seedlings at 80 ˚C for 1 week, the dry weight (DW) of the roots, as well as shoots, were recorded and the root DW was used to standardize the yeast numbers present on the blue lupin roots after treatment (Moller et al., 2016). In addition, the root and shoot DW of the seedlings were recorded for later use.

2.4.3. Inoculation of wheat with yeast strains

To inoculate wheat seeds with the yeast strains (Table 3.1), the surface sterilised seeds (n = 100) were submerged for 2 min in either sterile PSS (n = 28) or in the suspensions of either *H. zeae* CAB 1119 (n = 28), *P. laurentii* CAB 91 (n = 28), or *S. podzolica* CAB 1199 (n = 28). Subsequently, the seeds were dried under sterile conditions in a biosafety cabinet (LabAire) for 2 h. Thereafter, seeds (n = 25 per treatment) were planted onto MS agar and allowed to germinate in the dark for 4 d at

23 ˚C. The remaining seeds (n = 3 per treatment) were used to determine the number of yeast cells present on the seeds directly after treatment by rinsing each seed in sterile PSS. The resulting suspensions were subsequently diluted serially and the dilutions were spread plated onto YM agar. Following 2 d of incubation at 26 ˚C the colonies were enumerated and the DW of the seeds was used to standardize the yeast numbers present on the seeds after treatment. Following germination, wheat seedlings of uniform size (n = 10 per treatment) were planted in pots containing *ca.* 800 g of the sterile acid-washed silica sand. Three remaining wheat seedlings of each treatment were subsequently dried for 1 week at 80 ˚C and the DW of the roots and shoots were recorded for later use.

2.4.4. Plant growth and photosynthetic measurements

Following planting, both blue lupin and wheat were cultivated for one and two months in a glasshouse under the same conditions as given in section 2.4.2. During the growth period, 50 mL of a modified Long Ashton nutrient solution that had a low P content (10 µ*M;* Kleinert et al., 2014; Le Roux et al., 2014; Thuynsma et al., 2014a, 2014b) in the form of Na_2HPO_4 and NaH_2PO_4 (Merck) was administered to each plant on a weekly basis. In addition, each plant received 50 mL sterile dH_2O once a week. After one and two months of growth, and prior to harvesting, the maximum rate of CO_2 assimilation under light saturating conditions (A_{max}), rate of stomatal conductance to CO_2 (G_s), substomatal CO_2 (C_i), transpiration (E), and leaf dark respiration (Rd) were measured in the youngest fully expanded leaf of both blue lupin and wheat plants (n = 5 per treatment per month) with a portable infrared gas analyser (LI-6400XT, LI-COR Inc., Lincoln, NE, USA). Environmental conditions within the leaf chamber were as follows: a photosynthetic photon flux density of 1500 µmol m^{-2} s^{-1}, relative humidity of 36%, leaf vapour pressure deficit of 1.83 kPa, flow rate of 400 µmol s^{-1}, reference CO_2 level of 400 ppm and leaf temperature of 24 ˚C. Since the area of both blue lupin and wheat leaves are smaller than the area of the leaf chamber (6 cm^2), the leaves subjected to photosynthetic measurements were collected and the millimetre graph paper method (Pandey and Singh, 2011) was employed to determine the area of each leaf. The obtained leaf area was used to adjust all measured values, whereafter the photosynthetic water-use efficiency (PWUE) was calculated for each plant as A_{max}/ E (Medrano et al., 2015). In addition, the intrinsic water-use efficiency (IWUE) was

calculated as A_{max}/ G_s (Medrano et al., 2015) and potential productivity (Pp) was calculated as A_{max}/ Rd (Smith et al., 1995).

2.4.5. Harvesting and biomass parameters

Following photosynthetic measurements, blue lupin and wheat plants were harvested after one month (n = 5 per treatment) and two months (n = 5 per treatment) of growth. After the plants were gently uprooted, their roots were rinsed in sterile PSS. Subsequently, the fresh weight (FW) of the roots and shoots were recorded for both blue lupin and wheat plants, while the nodule numbers and nodule FW were noted for blue lupin plants only. Thereafter, the different plant organs were dried for 1 week at 80 ˚C in an oven. After the DW of the organs of both plants were recorded, the relative growth rate (RGR; Sibly and Vincent, 1997) of the roots, shoots, nodules (blue lupin only), and whole plant was calculated for the one month (RGR_{1MTH}) and two month (RGR_{2MTH}) growth period by taking the DW of the seedlings (see sections 2.4.2 and 2.4.3) as initial DW values. The RGR was also calculated for the different organs of blue lupin and wheat for the growth period that occurred between the first and second harvest (RGR_{BTWN}) by taking the DW of the one-month-old plants as the initial DW values. In addition, the fraction of new root biomass partitioned into nodules and new roots over a growth period, *i.e.* the below-ground allocation (BGA), was calculated using the equation given by Bazzaz (1997) for the one-month and two-month-old blue lupin plants.

2.4.6. Numbers of yeast symbionts in the rhizosphere

To determine the number of yeast cells present in the rhizosphere of each blue lupin and wheat plant, the PSS wherein the roots were rinsed (see section 2.4.5.) were serially diluted and the dilutions were plated onto thymine-mineral-vitamin (TMV) agar plates (Cornelissen et al., 2003). After 1 week of incubation at 26 ˚C, the yeast colonies present on the plates were counted, whereafter yeast isolates were obtained by randomly selecting colonies from the plates using the Harrison's disc method (Harrigan and McCance, 1976).

Following purification on YM agar, the yeast isolates were subjected to restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the ribosomal gene cluster to classify them according to their RFLP profiles.

For this purpose, the isolates were cultured for 16 h at 26 ˚C in 10 mL YM broth on a TC-7 tissue culture roller drum set to 60 rpm, which was followed by the extraction of genomic DNA (gDNA) as described by Vreulink et al. (2010). Subsequently, the ITS region of each isolate was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; Inqaba Biotechnical Industries, Pretoria, South Africa) (White et al., 1990). The 50 µL PCR reaction mixture contained 25 µL of a 2x master mix (ThermoFisher Scientific Inc., Waltham, MA, USA), 1.5 µL of each primer (10 µmol/ L), 21 µL sterile nuclease-free dH_2O (ThermoFisher Scientific Inc.) and 1 µL gDNA. Amplification was performed in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA), where the amplification program comprised an initial denaturation for 3 min at 95 °C, 30 cycles of denaturation for 45 s at 95 °C, annealing for 45 s at 52 °C and extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. The RFLP profiles were obtained by digesting the amplified ITS regions with the restriction endonucleases *Hin6I*, *HinfI* and *MspI* according to the specifications of the manufacturer (ThermoFisher Scientific Inc.). Subsequently, the fragments were separated on a 2% (w/v) agarose gel (Lonza Rockland Inc., Rockland, ME, USA) that contained 1% (w/v) ethidium bromide (Sigma-Aldrich). The obtained banding patterns were compared to a 100 bp DNA Ladder (ThermoFisher Scientific Inc.), as well as to the banding patterns of the yeast strains used to inoculate the plants.

Subsequently, the D1/ D2 domain of the large subunit ribosomal DNA (rDNA) of three representatives of each RFLP profile was amplified using PCR in conjunction with the universal primers F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3') and LR3 (5'-GGTCCGTGTTTCAAGACGG-3'; Inqaba Biotechnical Industries; Fell et al., 2000). The 50 µL PCR reaction mixtures were set up as described above and amplification was performed in a thermal cycler (model 2720; Applied Biosystems). Amplification parameters encompassed an initial denaturation for 3 min at 95 °C, which was followed by 36 cycles of denaturation for 30 s at 95 °C, annealing for 45 s at 58 °C and extension for 1 min at 72 °C, whereafter a final extension ensued for 7 min at 72 °C. To obtain the D1/ D2 nucleotide sequences of the isolates, an ABI3130xl genetic analyser (Applied Biosystems) was employed and subsequently the sequences were compared to those on the GenBank database (http://www.ncbi.nlm.nih.gov/blast) using the BLAST tool. Thereafter, the identity of the isolates together with their RFLP

profiles were used to calculate the relative numbers of the yeast strains occurring in the rhizospheres of the plants.

2.5. Statistical analyses

Where possible data are presented as mean ± standard error (SE) and normality tests were performed using a Shapiro-Wilk's test. Even though the data had a non-normal distribution, multivariate analysis of variance (MANOVA) was used to assess the effects and interactions of the yeast treatments on average germination, MGT and MSG, since analysis of variance (ANOVA) and MANOVA are known to be robust against the violation of the assumption of a normal distribution (Schmider et al. 2010). For the same reason, the effects and interactions of the yeast treatments on blue lupin and wheat vigour was assessed using ANOVA. The datasets obtained for plant growth and photosynthetic parameters was analysed with ANOVA. Means of all MANOVA and ANOVA analyses were compared at $p < 0.05$ using a *post hoc* Fisher's LSD multiple range test. All analyses were conducted with Statistica v. 12 (Dell Inc., Tulsa, OK, USA).

3. Results

3.1. Plant growth promoting traits of yeast strains

Analyses of the plant growth promoting traits of the yeast strains revealed that all of the yeasts possessed two or more of these traits (Table 3.2). The lowest ACC deaminase activity was detected in the protein extracts of *S. podzolica* CAB 1199, while the highest activity was measured in the extracts of *H. zeae* CAB 1119. Similarly, *H. zeae* CAB 1119 produced the highest quantity of IAA, while IAA production by *P. laurentii* CAB 91 was the lowest. Of the yeast strains, *S. podzolica* CAB 1199 solubilised the least PO_4^{3-} whereas *H. zeae* CAB 1119 solubilised the most PO_4^{3-}. In contrast, *H. zeae* CAB 1119 was incapable of decarboxylating any of the three amino acids, while *P. laurentii* CAB 91 decarboxylated arginine, lysine and ornithine to a greater extent than the positive control (data not shown). None of the yeast strains produced detectable levels of siderophores on the O-CAS agar. Only *H. zeae* CAB 1119 and *P. laurentii* CAB 91 was able to solubilise Zn.

Table 3.2. Plant growth promoting traits of *Hannaella zeae* CAB 1119, *Papiliotrema laurentii* CAB 91 and *Saitozyma podzolica* CAB 1199, *i.e.* the expression of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, production of the phytohormone indole-3-acetic acid (IAA), the solubilisation of phosphate (PO_4^{3-}) in the National Botanical Research Institute's phosphate growth medium (NBRIP) or in Pikovskaya's medium (PVK), possible polyamine production through decarboxylation of arginine, lysine and ornithine, the production of siderophores, and solubilisation of zinc (Zn).

Plant growth promoting trait	*H. zeae* CAB 1119	*P. laurentii* CAB 91	*S. podzolica* CAB 1199
ACC deaminase activity (nmol α-ketobutyrate mg^{-1} protein h^{-1})	1277.67	318.31 [a]	117.15
IAA production (µg log^{-1} cells ± SE) [µg mL^{-1} ± SE]	6.68 ± 1.14 [56.29 ± 2.70]	3.35 ± 0.14 [25.03 ± 1.70] [a]	3.41 ± 0.15 [31.24 ± 1.06]
PO_4^{3-} solubilisation in NBRIP (mg log^{-1} cells) [mg L^{-1}]	7360 [56.16]	1315 [8.66]	461 [3.05]
PO_4^{3-} solubilisation in PVK (mg log^{-1} cells) [mg L^{-1}]	4735 [39.90]	4301 [36.53]	2568 [20.85]
Arginine decarboxylation [b]	-	+++	w
Lysine decarboxylation [b]	-	++	-
Ornithine decarboxylation [b]	-	++	-
Siderophore production [b]	-	-	-
Zn solubilisation [b]	+	w	-

[a] Determined by Moller et al. (2016)

[b] Negative (-), weak (w), moderate (+), strong (++), exceptional (+++)

3.2. Effect of yeast strains on blue lupin germination and vigour

Treating blue lupin seeds with suspensions of *H. zeae* CAB 1119 and *P. laurentii* CAB 91 resulted in increased seed germination in comparison to that of the control seeds (Fig. 3.1a), while *S. podzolica* CAB 1199 had no effect on germination.

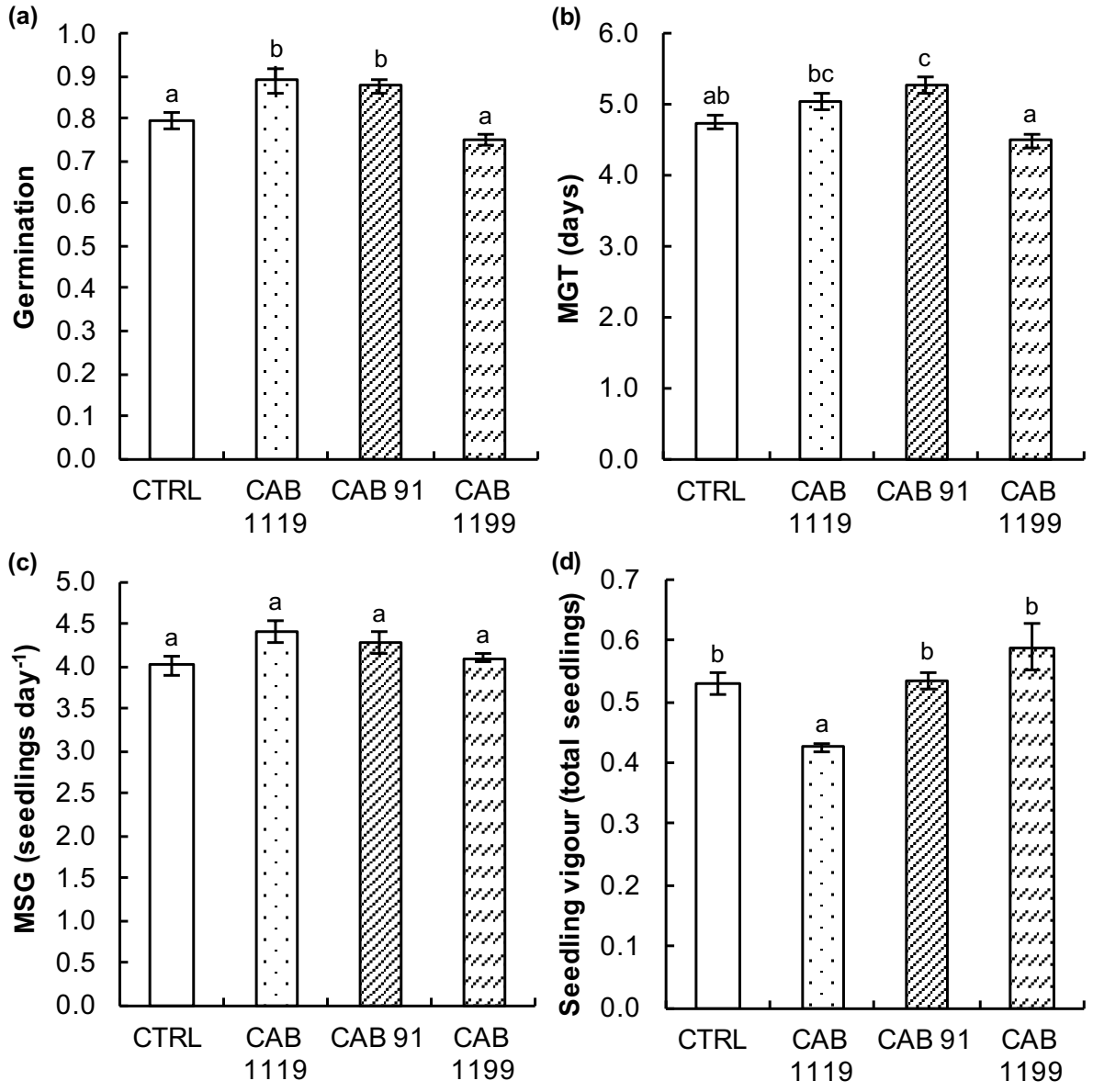

Fig. 3.1. Average germination of *Lupinus angustifolius* L., seeds **(a)** treated with either sterile PSS (CTRL) or log 8 cells mL^{-1} suspensions of either *Hannaella zeae* CAB 1119 (CAB 1119), *Papiliotrema laurentii* CAB 91 (CAB 91) or *Saitozyma podzolica* CAB 978 (CAB 978). Treated seeds (n = 100 per treatment) were dried for 2 h under sterile conditions and planted on MS agar. After 7 d of germination at 23 ˚C, seedling germination was evaluated according to the

guidelines of the International Seed Testing Association (ISTA, 1999). The average time for a seed to germinate (mean germination time [MGT]) **(b)** and the index of velocity of germination (Maguire's Speed of Germination [MSG]) **(c)** were calculated from the germination data. Vigour of *L. angustifolius* seeds **(d)**, treated with sterile PSS (CTRL) or log 8 cells mL^{-1} of CAB 1119, CAB 91 or CAB 1199, were assessed using the cold test method (Hampton and TeKrony, 1995). After treatment seeds (n = 200 per treatment) were allowed to dry for 2 h under sterile conditions before commencement of the cold test method. After 12 d, vigour was evaluated as stipulated by Hampton and TeKrony (1995). Bars represent the treatment means, while the error bars denote 1 standard error. The different letters are indicative of significant differences between the treatments ($p < 0.05$).

The MGT of blue lupin was increased when seeds were treated with *P. laurentii* CAB 91 in comparison to seeds treated with PSS (Fig. 3.1b). In contrast, the MGT of *H. zeae* CAB 1119 and *S. podzolica* CAB 1199 treated blue lupin seeds was the same as that of the control seeds. None of the yeast treatments had an effect on the MSG of blue lupin seeds (Fig. 3.1c). The vigour of blue lupin seeds treated with *P. laurentii* CAB 91 and *S. podzolica* CAB 1199 remained similar to that of seeds treated with PSS (Fig. 3.1d), while the seeds treated with *H. zeae* CAB 1119 resulted in reduced vigour.

3.3. Influence of yeast strains on germination and vigour of wheat

When wheat seeds were treated with *H. zeae* CAB 1119 and *S. podzolica* CAB 1199 the germination of these seeds were lower than that of the control seeds (Fig. 3.2a), while *P. laurentii* CAB 91 had no effect on the germination of wheat seeds. The MGT of wheat was reduced when seeds were treated *H. zeae* CAB 1119 (Fig. 3.2b). In contrast, the MGT of seeds treated with *P. laurentii* CAB 91 and *S. podzolica* CAB 1199 remained similar to that of the control seeds. The MSG of wheat seeds was lowered by *P. laurentii* CAB 91 and *S. podzolica* CAB 1199, whereas *H. zeae* CAB 1119 had no effect on this index when compared to seeds treated with PSS (Fig. 3.2c). In comparison to control seeds, wheat vigour was increased when seeds were treated with *P. laurentii* CAB 91 and *H. zeae* CAB 1119 (Fig. 3.2d), while vigour was unaffected by *S. podzolica* CAB 1199.

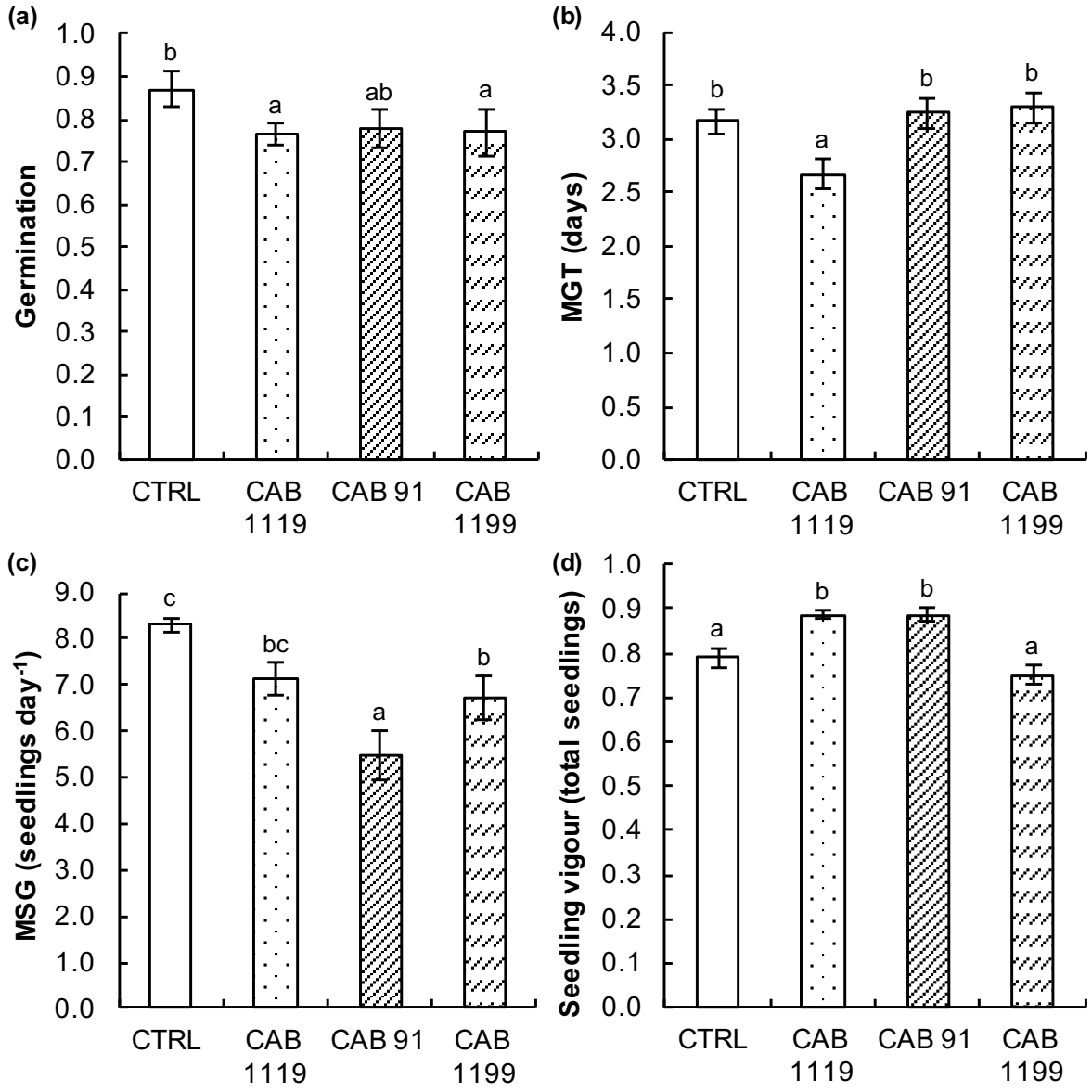

Fig. 3.2. Average germination of *Triticum aestivum* L. seeds after 7 d of germination at 23 ˚C **(a)**. Seeds were either treated with sterile PSS (CTRL) or with suspensions of log 8 cells mL^{-1} of either *Hannaella zeae* CAB 1119 (CAB 1119), *Papiliotrema laurentii* CAB 91 (CAB 91), or *Saitozyma podzolica* CAB 1199 (CAB 1199). The seeds (n = 100 per treatment) were dried under sterile conditions for 2h, which was followed by planting on MS agar. The guidelines given by the International Seed Testing Association (ISTA) were used to evaluate seedling germination (ISTA, 1999). The germination data was subsequently used to calculate the average time for a seed to germinate (mean germination time [MGT]) **(b)** and the index of velocity of germination (Maguire's Speed of Germination [MSG]) **(c)**. The cold test method (Hampton and TeKrony, 1995) was used to evaluate the vigour of *T. aestivum* seeds **(d)** treated with either sterile PSS (CTRL) or log 8 cells mL^{-1} suspensions of CAB 1119, CAB 91 or CAB 1199 that were dried for 2 h under sterile conditions. Vigour of the seedlings (n = 200

per treatment) was evaluated after 12 d as prescribed by Hampton and TeKrony (1995). Treatment means are given by the bars and the error bars represents 1 standard error. Significant differences between the treatments ($p < 0.05$) are indicated by the different letters.

3.4. Yeast symbionts present in rhizosphere

The number of *H. zeae* CAB 1119 cells present in the rhizosphere of one-month-old blue lupin and wheat was lower than the initial number of cells of this yeast strain on blue lupin roots and wheat seeds (Table 3.3). In contrast, after two months of growth the number of *H. zeae* CAB 1119 cells in the rhizosphere of blue lupin, as well as in that of wheat, was similar to the initial number of cells present on blue lupin roots and wheat seeds after treatment. The number of *P. laurentii* CAB 91 cells in the rhizosphere of one-month-old blue lupin plants was lower than the initial numbers of this yeast on the roots of blue lupin, while the number of this yeast present in the rhizosphere of two-month-old plants was similar to the initial numbers present on blue lupin roots. In contrast, the number of *P. laurentii* CAB 91 cells present in the rhizosphere of one-month-old and two-month-old wheat plants remained similar to the number of cells present on wheat seeds directly after treatment. Although *S. podzolica* CAB 1199 was present on blue lupin roots and wheat seeds after treatment, this yeast was not recovered from the rhizosphere of either one-month-old or two-month-old blue lupin and wheat plants. Since this yeast was not present in the rhizosphere of either plant, none of the growth and photosynthesis data of *S. podzolica* CAB 1199 treated plants can be attributed to the presence of this yeast and are thus excluded from subsequent data sets (Fig. 3.3 and 3.4, as well as Table 3.4 and 3.5).

3.5. Effect of yeast strains on biomass of blue lupin and wheat

Treatment of blue lupin with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 had no effect on the root, shoot and total plant biomass of one-month-old plants in comparison to control plants (Fig. 3.3a). Similarly, the root biomass of two-month-old blue lupin plants was unaffected by *H. zeae* CAB 1119 and *P. laurentii* CAB 91 when compared to the control (Fig. 3.3b). In contrast, shoot and total plant biomass of two-month-old blue lupin plants was increased by *H. zeae* CAB 1119, while *P. laurentii* CAB 91 had no effect on the shoot and total plant biomass compared to that of control plants. Nodule biomass and BGA of blue lupin plants were unaltered in the presence of any of the yeast strains after one month and two months of growth (data not shown).

Table 3.3. Numbers of *Hannaella zeae* CAB 1119, *Papiliotrema laurentii* CAB 91 and *Saitozyma podzolica* CAB 1199 present on blue lupin roots (Initial blue lupin) and wheat seeds (Initial wheat) directly after treatment with log 8 cells mL^{-1} suspensions of the yeast strains, as well as the numbers of these yeast strains present in the rhizosphere of blue lupin and wheat after one month and two months of growth under controlled conditions. Yeast numbers were determined using serial dilution plates with yeast extract-malt extract agar prepared from the physiological saline solution (PSS) used to rinse the roots or seeds of the respective plants. The colony forming units were divided by the dry weight of the roots or seeds, and the obtained values were log transformed resulting in log cells g^{-1} root or log cells g^{-1} seed. All values represent treatment means (n = 5) ± standard error. Different lettering represents significant differences ($p < 0.05$) between the numbers for each yeast strain on blue lupin, while prime lettering indicates significant differences ($p < 0.05$) between the numbers of each yeast strain on wheat.

Yeast treatment	Initial blue lupin (log cells g^{-1} root)	Initial wheat (log cells g^{-1} seed)	Blue lupin rhizosphere (log cells g^{-1} root)		Wheat rhizosphere (log cells g^{-1} root)	
			1 month	2 month	1 month	2 month
H. zeae CAB 1119	8.70 ± 0.033 a	7.19 ± 0.057 a’	5.53 ± 0.164 b	7.93 ± 0.251 a	6.16 ± 0.223 b’	6.74 ± 0.050 a’
P. laurentii CAB 91	8.17 ± 0.094 a	6.89 ± 0.015 a’	6.58 ± 0.107 b	8.21 ± 0.220 a	6.29 ± 0.127 a’	7.04 ± 0.404 a’
S. podzolica CAB 1199	8.30 ± 0.022	5.44 ± 0.092	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]

[a] After one and two months of growth, *S. podzolica* CAB 1199 was not detected (N.d.) in the rhizosphere of either plant.

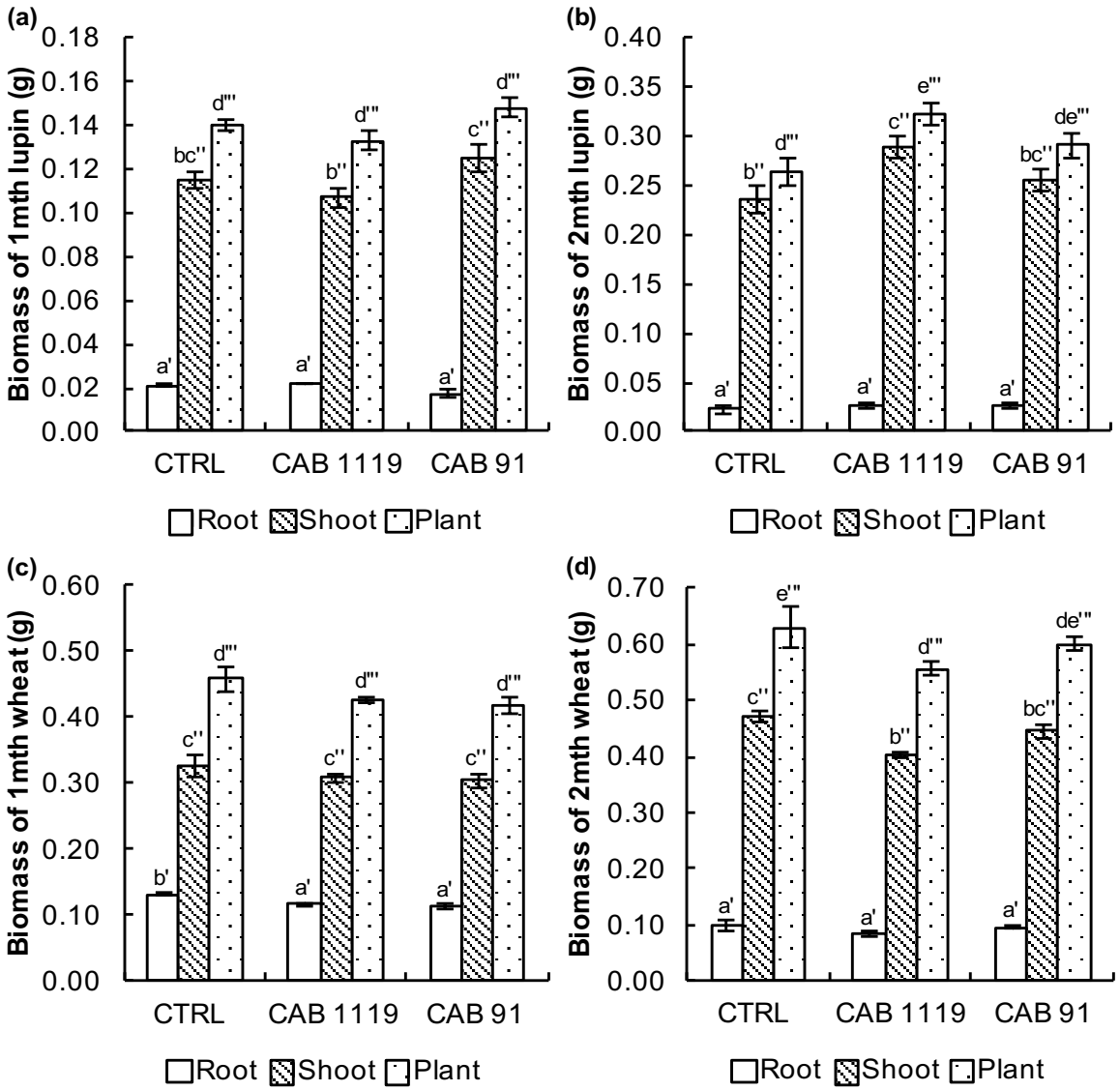

Fig. 3.3. Biomass parameters of *Lupinus angustifolius* L. **(a and b)** and *Triticum aestivum* L. **(c and d)** after one (1mth) **(a and c)** and two (2mth) **(b and d)** months of growth in a low phosphorous (10 µM) environment. Plants (n = 5 per treatment) were treated with either sterile PSS (CTRL) or log 8 cells mL^{-1} suspensions of either *Hannaella zeae* CAB 1119 (CAB 1119) or *Papiliotrema laurentii* CAB 91 (CAB 91). After each growth period plants were analysed for their root, shoot and total biomass. Bars denote the means of each treatment (n = 5) and 1 standard error is represented by the error bars. Different lettering shows significant differences between the treatments ($p < 0.05$) and prime lettering indicates that comparisons are made within the same parameter.

When wheat was treated with *H. zeae* CAB 1119 and *P. laurentii* CAB 91, the root biomass of one-month-old plants (Fig. 3.3c) was reduced compared to the control. In contrast, after one month of growth the shoot and total biomass of wheat plants treated with the yeast strains was unchanged in comparison to that of the control plants.

Similarly, root biomass of two-month-old wheat treated with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 was not significantly different to that of the control (Fig. 3.3d). In contrast, the shoot and total plant biomass of two-month-old wheat was reduced when treated with *H. zeae* CAB 1119, while shoot and total plant biomass of plants treated with *P. laurentii* CAB 91 was similar to the control after two months of growth.

3.6. Yeast impact on lupin and wheat RGR

The root RGR_{1MTH}, shoot RGR_{1MTH} and plant RGR_{1MTH} of blue lupin treated with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 was unchanged compared to that of the control plants (Fig. 3.4a). Likewise, the root RGR_{2MTH}, shoot RGR_{2MTH} and plant RGR_{2MTH} of blue lupin treated with *P. laurentii* CAB 91 was similar to that of the control (Fig. 3.4b). In contrast, *H. zeae* CAB 1119 increased the shoot RGR_{2MTH} and plant RGR_{2MTH} of blue lupin when compared to the control, while this yeast strain had no effect on the root RGR_{2MTH}. Treating blue lupin with *H. zeae* CAB 1119 had no effect on root RGR_{BTWN} in comparison to the control (Fig. 3.4c), while *P. laurentii* CAB 91 increased the root RGR_{BTWN} of blue lupin compared to that of control plants. Furthermore, the shoot RGR_{BTWN} and plant RGR_{BTWN} of blue lupin was increased when the plants were treated with *H. zeae* CAB 1119 and *P. laurentii* CAB 91. None of the yeast treatments had an effect on the nodule RGR_{1MTH}, nodule RGR_{2MTH} and nodule RGR_{BTWN} of blue lupin (data not shown).

Treating wheat with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 decreased root RGR_{1MTH} compared to the control, while the yeast treatments had no effect on shoot RGR_{1MTH} and plant RGR_{1MTH} (Fig. 3.4d). Similarly, root RGR_{2MTH} was unaffected by the yeast treatments in comparison to that of control plants (Fig. 3.4e). In contrast, *H. zeae* CAB 1119 reduced the shoot RGR_{2MTH} and plant RGR_{2MTH} of wheat, while *P. laurentii* CAB 91 had no effect on these parameters when compared to the control. Although negative values where obtained for root RGR_{BTWN} of wheat plants, the root RGR_{BTWN} of wheat treated with *P. laurentii* CAB 91 (- 3.549 ± 0.822; $p < 0.05$) was less negative than that of control plants (- 9.788 ± 2.645), while the root RGR_{BTWN} of *H. zeae* CAB 1119 treated plants (- 8.591 ± 1.431) was similar to that of the control ($p > 0.05$). Similarly, the shoot RGR_{BTWN} and plant RGR_{BTWN} of wheat treated with *H. zeae* CAB 1119 was not significantly different to that of the control plants (Fig. 3.4f), while *P. laurentii* CAB 91 increased shoot RGR_{BTWN} and plant RGR_{BTWN} of wheat.

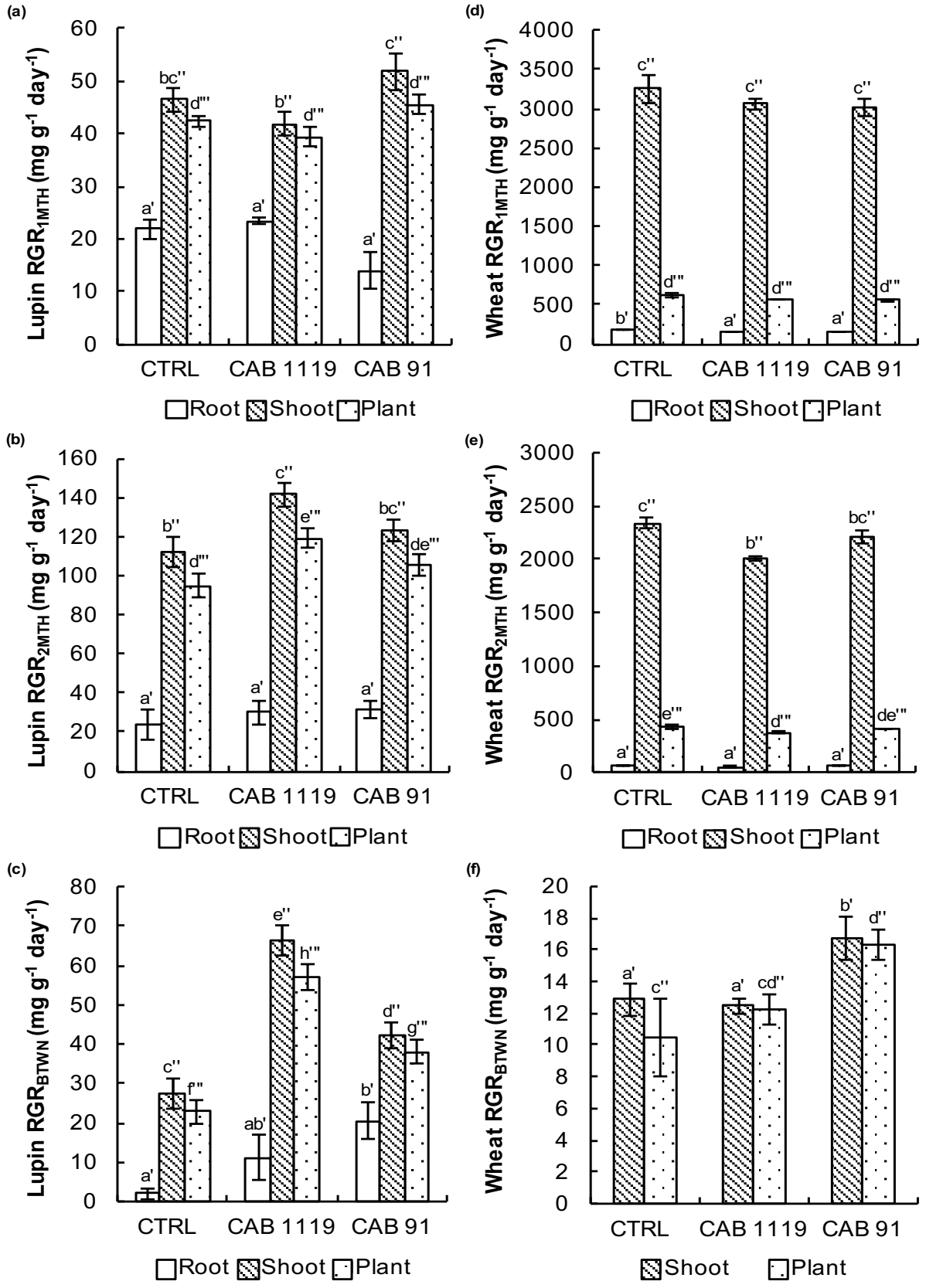

Fig. 3.4. Relative growth rates (RGR) of *Lupinus angustifolius* L. **(a, b and c)** and *Triticum aestivum* L. **(d, e and f)** treated with sterile PSS (CTRL) or with suspensions of log 8 cells mL^{-1} of either *Hannaella zeae* CAB 1119 (CAB 1119) or *Papiliotrema laurentii* CAB 91 (CAB

91). The RGR of one-month-old plants (RGR_{1MTH}) was calculated for the root, shoot and total plant for both *L. angustifolius* **(a)** and *T. aestivum* **(d)**. Dry weights of two-month-old plants was used to calculate the RGR for the two-month growth period (RGR_{2MTH}) for the root, shoot and total plant of *L. angustifolius* **(b)** and *T. aestivum* **(e)**. In addition, the RGR of root, shoot and total plant was calculated for the one-month growth period between the first and second month of growth (RGR_{BTWN}) for *L. angustifolius* **(c)**, while the shoot and total plant RGR_{BTWN} was calculated for *T. aestivum* **(f)**. The means of each treatment (n = 5) is indicated by the bars, while the error bars denotes 1 standard error. Significant differences ($p < 0.05$) between the treatments are represented by the different letters, while prime letters signify comparisons within the same parameter.

3.7. Effect of yeasts on photosynthetic parameters of blue lupin and wheat

When compared to the control, the A_{max} of one-month-old blue lupin plants treated with *H. zeae* CAB 1119 was lower, while *P. laurentii* CAB 91 had no effect on this parameter (Table 3.4). After two months of growth, A_{max} was increased by *H. zeae* CAB 1119 and *P. laurentii* CAB 91 in comparison to that of control plants. None of the yeast treatments had an effect on G_s of one-month-old blue lupin plants. In contrast, *H. zeae* CAB 1119 decreased G_s after two months of growth in comparison to the control, while *P. laurentii* CAB 91 had no effect on this parameter. The C_i of one-month-old blue lupin plants was reduced by *P. laurentii* CAB 91 and increased by *H. zeae* CAB 1119 relative to that of control plants. In contrast, after two months of growth the C_i of *H. zeae* CAB 1119 and *P. laurentii* CAB 91 treated blue lupin plants was decreased when compared to the control. None of the yeast treatments had an effect on E or Rd of blue lupin after one and two months of growth. The PWUE and IWUE of one-month-old blue lupin plants were decreased by *H. zeae* CAB 1119, while these parameters were increased by *P. laurentii* CAB 91 relative to that of control plants. While the PWUE of two-month-old blue lupin plants treated with *H. zeae* CAB 1119 was higher than that of the control plants, this parameter was not influenced by *P. laurentii* CAB 91. After two months of growth, the IWUE of blue lupin was increased by both yeast treatments. The only yeast treatment to influence Pp of blue lupin was *H. zeae* CAB 1119, since this yeast treatment decreased Pp of one-month-old plants, while it increased Pp of two-month-old plants.

Table 3.4. Photosynthetic parameters, *i.e.* the maximum rate of CO_2 assimilation under light saturating conditions (A_{max}), rate of stomatal conductance to CO_2 (G_s), substomatal CO_2 (C_i), transpiration (E), leaf dark respiration (Rd), photosynthetic water use efficiency (PWUE), intrinsic water use efficiency (IWUE), and potential productivity (Pp), of *Lupinus angustifolius* L. (blue lupin) treated with sterile PSS (CTRL) or log 8 cells mL^{-1} suspension of *Hannaella zeae* CAB 1119 (CAB 1119) or *Papiliotrema laurentii* CAB 91 (CAB 91). After one and two months of growth in a low phosphorous environment photosynthetic measurements were taken of the youngest fully expanded leaf of each plant. Values denotes treatment means (n = 5) ± standard error (SE), while different lettering indicates significant differences ($p < 0.05$) between the treatments in each parameter. Prime lettering signifies that comparisons are made within each month.

Parameter	One-month-old blue lupin			Two-month-old blue lupin		
	CTRL	CAB 1119	CAB 91	CTRL	CAB 1119	CAB 91
A_{max} (µmol CO_2 m^{-2} s^{-1} ± SE)	14.4 ± 1.18 a'	4.95 ± 0.182 b'	13.0 ± 1.37 a'	4.43 ± 0.10 a''	6.86 ± 0.34 b''	6.69 ± 0.11 b''
G_s (mol CO_2 m^{-2} s^{-1} ± SE)	0.08 ± 0.0003 a'	0.07 ± 0.003 a'	0.06 ± 0.01 a'	0.14 ± 0.02 a''	0.08 ± 0.01 b''	0.12 ± 0.02 ab''
C_i (µmol CO_2 m^{-2} s^{-1} ± SE)	207 ± 13.3 a'	311 ± 5.31 b'	161 ± 0.03 c'	355 ± 3.25 a''	300 ± 7.73 b''	327 ± 10.3 c''
E (mmol H_2O m^{-2} s^{-1} ± SE)	3.51 ± 0.04 a'	3.23 ± 0.20 a'	2.55 ± 0.30 a'	3.36 ± 0.38 a''	2.36 ± 0.41 a''	3.56 ± 0.72 a''
Rd (µmol CO_2 m^{-2} s^{-1} ± SE)	2.50 ± 0.05 a'	2.89 ± 0.15 a'	2.92 ± 0.89 a'	5.05 ± 0.41 a''	3.69 ± 1.07 a''	4.08 ± 0.47 a''
PWUE (mmol CO_2 mol^{-1} H_2O ± SE)	4.12 ± 0.39 a'	1.56 ± 0.15 b'	5.12 ± 0.06 c'	1.36 ± 0.12 a''	3.12 ± 0.40 b''	2.12 ± 0.40 a''
IWUE (mmol CO_2 mol^{-1} H_2O ± SE)	174 ± 13.6 a'	70.2 ± 5.58 b'	221 ± 2.01 c'	34.0 ± 3.28 a''	87.5 ± 8.38 b''	60.0 ± 10.7 c''
Pp (± SE)	5.75 ± 0.37 a'	1.73 ± 0.15 b'	5.58 ± 1.23 a'	0.89 ± 0.05 a''	2.39 ± 0.60 b''	1.70 ± 0.17 ab''

Treating wheat with *H. zeae* CAB 1119 reduced A_{max} after one month of growth (Table 3.5), while *P. laurentii* CAB 91 had no effect on this parameter in comparison to control plants. In contrast, two-month-old wheat plants treated with *H. zeae* CAB 1119 had a higher A_{max} than control plants, whereas this parameter was unaffected by *P. laurentii* CAB 91. Although both yeast treatments reduced G_s of one-month-old wheat plants, after two months of growth this parameter was unchanged by the yeast treatments. Treatment of wheat with the yeast strains had no effect on C_i after one month of growth when compared to that of the control plants, while C_i was reduced by *H. zeae* CAB 1119 and *P. laurentii* CAB 91 after two months of growth. After one month of growth E was decreased by both yeast treatments, while this parameter of two-month-old wheat plants treated with the yeast strains was similar to that of the control. Treating wheat plants with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 resulted in reduced Rd after one month of growth, while Rd was increased by *H. zeae* CAB 1119 in two-month-old wheat plants. The PWUE and IWUE of one-month-old wheat plants treated with *H. zeae* CAB 1119 and *P. laurentii* CAB 91 was similar to the control, whereas these yeast treatments increased PWUE and IWUE of two-month-old wheat plants. After the one-month growth period, the Pp of wheat plants was increased by *P. laurentii* CAB 91 compared to the control, while *H. zeae* CAB 1119 had no effect on this parameter. In contrast, both yeast treatments increased the Pp of wheat plants after two months of growth.

Table 3.5. Photosynthetic parameters, *i.e.* the maximum rate of CO_2 assimilation under light saturating conditions (A_{max}), rate of stomatal conductance to CO_2 (G_s), substomatal CO_2 (C_i), transpiration (E), leaf dark respiration (Rd), photosynthetic water use efficiency (PWUE), intrinsic water use efficiency (IWUE), and potential productivity (Pp), of *Triticum aestivum* L. (wheat). Plants were treated with sterile PSS (CTRL) or log 8 cells mL^{-1} suspensions of *Hannaella zeae* CAB 1119 (CAB 1119) or *Papiliotrema laurentii* CAB 91 (CAB 91), and cultivated for one and two months in a low phosphorous environment. Photosynthetic measurements were taken on the youngest fully expanded leaf of each plant. The values represent treatment means (n = 5) ± standard error (SE), while significant differences for each parameter ($p < 0.05$) between treatments are indicated by different lettering. Prime lettering distinguishes between comparisons that were made for each month.

Parameter	One-month-old wheat			Two-month-old wheat		
	CTRL	CAB 1119	CAB 91	CTRL	CAB 1119	CAB 91
A_{max} (µmol CO_2 m^{-2} s^{-1} ± SE)	6.33 ± 0.89 a'	3.73 ± 0.42 b'	4.67 ± 0.22 ab'	2.24 ± 0.05 a''	8.47 ± 0.44 b''	3.03 ± 0.40 a''
G_s (mol CO_2 m^{-2} s^{-1} ± SE)	0.04 ± 0.0001 a'	0.02 ± 0.01 b'	0.02 ± 0.001 b'	0.04 ± 0.02 ab''	0.06 ± 0.003 a''	0.03 ± 0.01 b''
C_i (µmol CO_2 m^{-2} s^{-1} ± SE)	224 ± 22.0 a'	190 ± 27.0 a'	148 ± 42.3 a'	321 ± 17.9 a''	234 ± 0.23 b''	278 ± 12.0 c''
E (mmol H_2O m^{-2} s^{-1} ± SE)	1.80 ± 0.02 a'	0.97 ± 0.23 b'	1.02 ± 0.06 b'	1.22 ± 0.40 a''	1.52 ± 0.08 a''	1.00 ± 0.27 a''
Rd (µmol CO_2 m^{-2} s^{-1} ± SE)	1.61 ± 0.03 a'	0.74 ± 0.13 b'	0.60 ± 0.06 b'	1.63 ± 0.29 a''	2.64 ± 0.14 b''	1.20 ± 2.09 a''
PWUE (mmol CO_2 mol^{-1} H_2O ± SE)	4.50 ± 0.45 a'	4.28 ± 0.59 a'	4.66 ± 0.49 a'	2.11 ± 0.55 a''	5.57 ± 0.02 b''	3.23 ± 0.42 c''
IWUE (mmol CO_2 mol^{-1} H_2O ± SE)	157 ± 22.4 a'	195 ± 27.8 a'	235 ± 24.9 a'	67.1 ± 18.2 a''	153 ± 0.21 b''	108 ± 12.7 c''
Pp (± SE)	3.92 ± 0.50 a'	5.24 ± 0.35 a'	8.15 ± 1.15 b'	1.45 ± 0.20 a''	3.22 ± 0.003 b''	2.52 ± 0.25 b''

4. Discussion

Yeasts are known to increase growth of a variety of plants (Amprayn et al., 2012; Cloete et al., 2009; Liu et al., 2016; Medina et al., 2004; Moller et al., 2016; Nassar et al., 2005; Silambarasan and Vangnai, 2017; Vassilev et al., 2001; Wang et al., 2013), however, it is unclear whether a single yeast strain can be employed to improve growth of crops used in break-crop and crop rotation systems. We hypothesised that *P. laurentii* CAB 91 may be used for such a purpose and thus compared its influence on germination, growth and photosynthesis of two crops, namely blue lupin and wheat, to that of two other rhizosphere yeasts, *i.e. H. zeae* CAB 1119 and *S. podzolica* CAB 1199. Assessment of PGP traits revealed that all three yeast strains possessed ACC deaminase activity, produced IAA and solubilized PO_4^{3-}. Furthermore, *H. zeae* CAB 1119 and *P. laurentii* CAB 91 solubilised Zn, while *P. laurentii* CAB 91 was the only yeast strain to decarboxylate all three amino acids. It is thus likely that *P. laurentii* CAB 91 produces multiple PAs, since decarboxylation of arginine and ornithine leads to the production of putrescine (Couée et al., 2004; Evans and Malmberg, 1989), which can further be converted to spermine and spermidine (Hamasaki-Katagiri et al., 1998; Imai et al., 2004), while decarboxylation of lysine results in the production of cadaverine (Bagni et al., 1986; Tomar et al., 2013). The results obtained during the present study indicate that all three yeast strains are potential plant growth promoting yeasts (PGPY).

Previous research has demonstrated that PGPY may influence the germination of different plants (Akhtyamova and Sattarova, 2013; Gaballah and Gomaa, 2004; Matić et al., 2014; Nakayan et al., 2013; New et al., 2013; Ramos-Garza et al., 2016; Shalaby and El-Nady, 2008). Similarly, during the present study *H. zeae* CAB 1119 increased the germination of blue lupin seeds. Yet, this yeast strain reduced the vigour of these seeds. Since reduced vigour may result in lower emergence under field conditions (ISTA, 2014), *H. zeae* CAB 1119 may not be a viable candidate to promote blue lupin germination in the field. Considering that *P. laurentii* CAB 91 increased the germination of blue lupin seeds, while seed vigour was unaffected, this yeast strain can possibly be used as a seed coating agent to improve blue lupin germination. Likewise, *P. laurentii* CAB 91 might serve as a seed coating agent to improve wheat vigour in the field, since this was the only yeast strain to increase wheat vigour without decreasing seed germination. The means whereby *P. laurentii* CAB 91 increased the germination

of blue lupin and the vigour of wheat seeds are unclear, since nothing is known about the mechanisms of action, except for indications that IAA production by a strain of *Rhodotorula mucilaginosa* may result in improved germination of *Brassica juncea* (L.) Czern. (Ramos-Garza et al., 2016). It is possible that the increased germination of blue lupin seeds treated with *P. laurentii* CAB 91 was due to the production of PAs by this yeast strain, since these growth regulators (Evans and Malmberg, 1989; Galston and Sawhney, 1990) can influence seed germination (Sinska, 1988). It is also likely that *P. laurentii* CAB 91 produces other hormones involved in seed germination, for instance gibberellins, since other PGPY are able to produce gibberellic acid (El-Tarabily, 2004). The increased vigour of wheat treated with *P. laurentii* CAB 91 is possibly due to the production of ACC deaminase by this yeast strain, since the cold test is a stress test (Bennett et al., 2004) that may lead to 'stress ethylene' production and ACC deaminase producing bacteria were found to increase seed germination under salt stress by reducing 'stress ethylene' (Jalili et al., 2009; Siddikee et al., 2015). It is unclear why *P. laurentii* CAB 91 had no influence on the vigour of blue lupin and the germination of wheat and future work is thus needed to elucidate the mechanisms behind this yeast's influence on seed germination and vigour.

In addition to assessing the effect of the yeast strains on germination and vigour, the impact of the yeast treatments on the germination speed of blue lupin and wheat seeds were determined by calculating two germination indices, *i.e.* MGT and MSG. For some of the yeast treatments we observed contrasting results between the two indices. This is not uncommon, since germination indices can yield inconsistent results (Brown and Mayer, 1988) and therefore it was recommended that more than one germination index should be used when determining germination rate. However, from our results it is evident that multiple indices should be used in future when evaluating the effect of yeasts on germination speed.

During the pot trial conducted in the present study, we evaluated whether the yeast strains could colonise the rhizosphere of blue lupin and wheat, thereby allowing these unicellular fungi to potentially impact the growth of the two plants. Considering that *S. podzolica* CAB 1199 was originally isolated from the rhizosphere, it is unexpected that this yeast was not present in the rhizosphere of either plant after one or two

months of growth. However, it is known that the rhizosphere competence of microorganisms, *i.e.* the ability to colonise and grow in the rhizosphere, varies (Schreiter et al., 2014) based on the ability of a microorganism to attach to the host root (Albareda et al., 2006) and to utilize the root exudates as substrates for growth (Lugtenberg and Kamilova, 2009). Since the original host plant of *S. podzolica* CAB 1199, *i.e. Eragrostis trichophora* (Table 3.1), is distantly related to wheat (Soreng et al., 2015) and even less related to blue lupin, it is probable that this yeast strain is competent to colonise the rhizosphere of *E. trichophora*, but not that of blue lupin and wheat. In contrast, the results obtained in the present study indicate that *H zeae* CAB 1119 and *P. laurentii* CAB 91 are capable to colonise the rhizosphere of both blue lupin and wheat.

Despite this competency, *H. zeae* CAB 1119 elicited different effects on the growth and photosynthetic parameters of the two plants. The increased shoot and total biomass of two-month-old blue lupin plants treated with this yeast, coincides with the greater shoot and total RGR of the plants during the same growth period. It seems evident that the increased photosynthetic rate (A_{max}) was driving the increased growth of these plants, since higher photosynthetic rates are known to result in greater biomass production (Ainsworth and Long, 2005; Long et al., 2006). The increased PWUE and IWUE of two-month-old blue lupin treated with *H. zeae* CAB 1119 is a consequence of an increased photosynthetic rate (Cloete et al., 2010b), in conjunction with an unchanged rate of water loss, as reflected in the transpiratory water loss (E) of these plants. The mechanism by which this yeast strain stimulated the photosynthetic rate of the blue lupin plants is unclear, but it has been suggested that yeast symbionts may induce a sink stimulation of photosynthesis, owing to their requirement of host-derived carbon (C) (Cloete et al., 2010b).

In spite of the increase in the photosynthetic rate of wheat treated with *H. zeae* CAB 1119, the reduced biomass production in these plants suggests a diversion in the allocation of photosynthetic products. In this regard, the treatment of wheat with *H. zeae* CAB 1119 may have stimulated the allocation of photosynthates towards the growth of this yeast (Miller et al., 2002), instead of being allocated for plant growth. This is supported by the increased dark respiration rate (Rd) of two-month-old wheat plants treated with *H. zeae* CAB 1119, which indicates that the metabolism of the

leaves was most likely geared towards increasing photosynthetic C gain (Cloete et al., 2010b), to satisfy the C-sink demand of this yeast. Furthermore, the higher ratio of photosynthesis to dark respiration, as reflected in the increased potential productivity (Pp), in the wheat plants treated with *H. zeae* CAB 1119 supports this contention of leaf metabolic attenuation, aimed at increasing C gain through photosynthesis. Although a higher photosynthesis rate, coupled with an unchanged transpiration rate, resulted in increased PWUE and IWUE, the metabolic attenuation of the leaves due to the C-sink demand of *H. zeae* CAB 1119 ultimately had a negative impact on wheat growth.

The increased root RGR, shoot RGR and total RGR of blue lupin treated with *P. laurentii* CAB 91 during the second month of growth can be ascribed to the increased photosynthetic rate of two-month-old blue lupin plants, since plant growth is affected by the rate of photosynthesis (Ainsworth and Long, 2005; Long et al., 2006). In the presence of *P. laurentii* CAB 91, the stomatal conductance to CO_2 (G_s) of two-month-old blue lupin plants was unaltered, while there was a reduction in intercellular CO_2 (C_i). This suggests that stomata of these plants were unaffected by the yeast treatment (Taub, 2010; Tezara et al., 1999), which is evidenced by the unchanged transpiration rate for two-month-old blue lupin treated with *P. laurentii* CAB 91. Although PWUE of two-month-old blue lupin treated with this yeast was similar to that of the control, IWUE was increased by this yeast treatment, since these plants had an elevated photosynthetic rate. The mechanism whereby *P. laurentii* CAB 91 increased the photosynthetic rate of blue lupin is possibly due to the production of PAs by this yeast, since exogenous application of these aliphatic compounds to roots is known to increase photosynthesis under different environmental stresses (Chen et al., 2011; Duan et al., 2008). This has been ascribed to increased accumulation of PAs in chloroplasts resulting in greater amounts of chlorophyll *a* (Shu et al., 2012) and photosystem II activity. Yet, the role of PAs in blue lupin photosynthesis is unclear and should thus be investigated in future to establish whether *P. laurentii* CAB 91 increased the photosynthetic rate of this plant through the production of PAs.

Although *P. laurentii* CAB 91 reduced the root growth of wheat after one month of cultivation, the root, shoot and plant biomass of two-month-old plants treated with this yeast was similar to that of the control. This increase in wheat biomass during the

second month of growth is evident in the increased root, shoot and total RGR observed for *P. laurentii* CAB 91 treated plants. Considering that photosynthetic rate of two-month-old wheat plants was unaffected by *P. laurentii* CAB 91, while PWUE and IWUE was increased during this period, it is apparent that transpiration rate had to be lower in these plants during this growth period. Since higher PWUE has been linked to increased biomass of crops (Pieters and Núñez, 2008), it is likely that the increased RGR of wheat during the second month of cultivation is due to the increase of PWUE and IWUE by *P. laurentii* CAB 91. In addition, there is an indication that *P. laurentii* CAB 91 lowered the stomatal conductance to CO_2 of two-month-old wheat, which could have contributed to increased PWUE and IWUE. It is possible that *P. laurentii* CAB 91 achieved this through the production of PAs, since it has been demonstrated that PAs can inhibit stomatal opening under saline conditions (Çavuşoğlu et al., 2007; Liu et al., 2000; Shi et al., 2010). However, future work is needed to elucidate the exact mechanisms whereby *P. laurentii* CAB 91 affects photosynthetic parameters in wheat, as well as in blue lupin.

5. Conclusions

Based on our findings, inferences on the suitability of the three yeast strains as potential bio-fertilisers of blue lupin and wheat can be made. Since *S. podzolica* CAB 1199 was not re-isolated from the rhizosphere of either plant during the pot trial, this yeast strain seems to be unsuitable for the use as a plant growth promoter for these crops. Although *H. zeae* CAB 1119 may act as a PGPY of blue lupin by increasing photosynthetic rate and PWUE, this yeast decreased seed vigour of the plant, thus negating its use as a seed coating agent for blue lupin. Additionally, using *H. zeae* CAB 1119 as a bio-fertiliser for wheat plants is not recommended, since this yeast reduced wheat germination and had no impact on wheat growth, while it stimulated the translocation of photosynthates to roots. In contrast, the results show that *P. laurentii* CAB 91 can be used as seed coating agent for both blue lupin and wheat, and that this yeast may also improve the growth of the plants by increasing photosynthetic rate and water relations. This indicates that *P. laurentii* CAB 91 may serve as bio-fertiliser of both blue lupin and wheat.

Since previous research revealed that *P. laurentii* CAB 91 increases efficiency of N-fixation in the nodules of blue lupin (Moller et al., 2016), it is possible that blue lupin

treated with this yeast may be conferred advantage in low N soils. Consequently, in break-crop and crop rotation systems with blue lupin and wheat, this yeast may be employed to increase the growth of both plants. However, future work is needed to assess the impact of *P. laurentii* CAB 91 on growth of blue lupin and wheat during field trials. Apart from elucidating the fundamental understanding of the symbioses between this yeast and its host plants, the mechanisms of action of photosynthetic stimulation by *P. laurentii* CAB 91 is of agricultural importance, as this is a factor that may potentially affect crop production. Elucidation of these functional interactions between other yeasts and their host plants would enable the efficient selection of beneficial yeast symbionts for the promotion of plant growth.

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Chapter 4 – General conclusions and future research

The green revolution helped to feed billions of people across the globe between the 1960s and 1980s (García-Fraile et al. 2017), due to the development and spread of technologies (Benbi 2017), such as the use of high yielding crop varieties, modern irrigation systems, pesticides and synthetic nitrogen (N) fertilisers. These technologies allowed for intensive crop production, thereby resulting in greater agricultural outputs. However, it is predicted that as a result of climate change, intensive crop production will be unable to meet the food demand of an ever-increasing population (FAO and ITPS 2015), which is estimated to reach approximately 9.6 billion people by 2050. These factors together with the need to reduce the reliance on N fertilisers (Vitousek et al. 1997; Smil 2000; Adesemoye and Kloepper 2009), has led to what is known as the 'new green revolution' (García-Fraile et al. 2017); an initiative aimed towards increasing agricultural output in a sustainable manner (Martin-Guay et al. 2018).

One sustainable agriculture practice that has been successfully used to increase yields of cereal crops, *e.g.* wheat (*Triticum aestivum* L.), while N fertiliser input is reduced, is break-crop and crop rotation systems (Kirkegaard et al. 2008; Angus et al. 2015). The success of these systems is mostly due to N being fixed by di-nitrogen (N_2) fixing plants, *e.g.* blue lupin (*Lupinus angustifolius* L.), which is cultivated as the alternative crop. Another promising avenue for improving crop yield in a sustainable manner is the use of plant growth promoting microorganisms (PGPM) as bio-fertilisers (Wezel et al. 2014; Timmusk et al. 2017). This is due to the knowledge that PGPM increase the growth and mineral nutrition of plants (Pellegrino et al. 2015; Çakmakçı et al. 2017), as well as elevate the tolerance of plants to abiotic stress (Khalafallah and Abo-Ghalia 2008; Çakmakçı et al. 2017). Although these bio-fertilisers may include bacteria, mycorrhizal fungi or yeasts, the latter is the least studied of the three.

Nevertheless, plant growth promoting yeasts (PGPY) are not only known to increase growth of their host plant (Mukherjee and Sen 2014; Fu et al. 2016; Liu et al. 2016; Silambarasan and Vangnai 2017), but also to improve germination (Matić et al. 2014; Ramos-Garza et al. 2016), nutrition (Morsy et al. 2014; Kang et al. 2015) and photosynthesis (Cloete et al. 2010) of the plant. Additionally, PGPY are able to form tripartite and quadripartite interactions with legumes, rhizobia and/or mycorrhizal fungi (Tuladhar and Subba Rao 1985; Singh et al. 1991; Vassilev et al. 2001; Fracchia et al. 2003; Medina et al. 2004; Azcon et al. 2010), resulting in increased plant growth.

Up until this study, however, it was unknown whether PGPY are able to form these symbioses with blue lupin and its root symbionts. In addition, to date the effect of a single yeast strain on more than one crop that is used in a break-crop or crop rotation system was unexplored.

During this study, the yeast *Papiliotrema laurentii* CAB 91, originating from the rhizosphere of free growing blue lupin (Chapter 2), was found to form a tripartite association with blue lupin and rhizobia, as well as a quadripartite symbiosis with this plant, rhizobia and mycorrhizal fungi. Plants partaking in the tripartite association had better growth and improved phosphorous (P) nutrition, while *P. laurentii* CAB 91 was found to positively influence biological N_2 fixation (BNF), since BNF efficiency was increased in the presence of this yeast. In the quadripartite association, growth and P nutrition of blue lupin was also increased, yet these plants followed a different N nutrition strategy to plants that formed the tripartite symbioses. The N nutrition of the former was indirectly affected by *P. laurentii* CAB 91, since this yeast stimulated mycorrhizal colonisation of the roots, which in turn resulted in greater nodulation and efficient growth on N resources. This was the first study to demonstrate that PGPY can positively influence the BNF of legumes and stimulate the efficient use of N resources for growth.

Considering these findings together with the role of blue lupin in break-crop and crop rotation systems, it was proposed that *P. laurentii* CAB 91 may influence these systems as a whole. Thus, the effect of this yeast on germination, growth, photosynthesis and vigour of blue lupin and wheat was investigated by comparing these effects to that of two other yeasts found in the rhizosphere of plants, *i.e. Hannaella zeae* CAB 1119 and *Saitozyma podzolica* CAB 1199 (Chapter 3). It was established that while all three yeast strains possess plant growth promoting (PGP) traits, *P. laurentii* CAB 91 exhibited the most of these traits. In addition, this yeast strain was the only microorganism evaluated in this study to potentially produce multiple polyamines (PAs). After the effect of the yeast strains on above-mentioned parameters was determined for both blue lupin and wheat, inferences were made on the suitability of these unicellular fungi to serve as bio-fertilisers for the plants. Since *S. podzolica* CAB 1199 was not re-isolated from the rhizosphere of blue lupin or wheat, it was recommended that this yeast strain should not be used as a bio-fertiliser.

Similarly, the usage of *H. zeae* CAB 1119 as a bio-fertiliser in a break-crop or crop rotation system was not endorsed, due to the germination and growth of wheat being reduced by this yeast strain. In contrast, it was concluded that *P. laurentii* CAB 91 might serve as a bio-fertiliser for both blue lupin and wheat, as a result of its positive effect on blue lupin and wheat germination, growth, photosynthesis and vigour.

Taken together, the results obtained from this study indicate that blue lupin might benefit from its association with *P. laurentii* CAB 91 in low N soils (Chapter 2). This can in turn positively influence wheat growth, since N uptake from soil by blue lupin is reduced under improved BNF (Chapter 2), which will result in larger quantities of N remaining in the soil. If this is coupled to additional blue lupin biomass being returned to the soil due to increased growth, it is evident that more N will be available in the soil for wheat growth. These aspects together with the ability of *P. laurentii* CAB 91 to influence the germination, growth and photosynthesis of both plants (Chapter 3), indicate that this yeast might not only affect the two plants separately, but also a break-crop or crop rotation system as a whole. Therefore, it seems likely that this yeast strain can serve as a bio-fertiliser for blue lupin and wheat in these systems.

It is, however, important to note that the results obtained with blue lupin and wheat in this study are preliminary, since all experiments were conducted under controlled conditions. Although pot trials are valuable for the initial study of plant interactions (Knight and Will 1971), results obtained in such trials are not always relatable to that observed in the field, since environmental conditions can vary considerably during field trials. Therefore, it is imperative that future studies with these crops include investigations on the impact of *P. laurentii* CAB 91 on germination, growth, nutrition and photosynthesis under field conditions. Although the results showed that *P. laurentii* CAB 91 can potentially influence a break-crop or crop rotation system as a whole, future research is needed to establish whether this occurs in the field when these systems are employed. Furthermore, the influence of *P. laurentii* CAB 91 on grain filling, grain nutrient content and yields were not evaluated in the present study and therefore future studies should also be aimed towards the investigation of these aspects. Considering that climate change will decrease crop production as a result of increased temperatures, drought and degraded soils, it is vital to determine whether *P. laurentii* CAB 91 can alleviate the negative effects of these abiotic stresses on plant

growth during field trials. Based on this yeast strain's PGP traits, it can be hypothesised that *P. laurentii* CAB 91 is able to ameliorate these effects by producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, increasing P and zinc (Zn) availability in soil via the solubilisation of these nutrients, and improving biomass as a result of indole-3-acetic acid (IAA) production. This hypothesis should be tested in future. In addition, the mechanisms underlying the ability of *P. laurentii* CAB 91 to affect nodulation and BNF of blue lupin, as well as to influence seed germination and photosynthesis of blue lupin and wheat, should be investigated.

The production of PAs by *P. laurentii* CAB 91 might be a mechanism that influenced the above-mentioned plant physiological processes, since PAs can affect nodulation (Vassileva and Ignatov 1999; López-Gómez et al. 2017), nitrogenase activity (López-Gómez et al. 2017), seed germination (Sinska 1988) and photosynthetic parameters (Duan et al. 2008; Groppa and Benavides 2008; Chen et al. 2011; Shu et al. 2012). Considering that the method used to detect PAs production by *P. laurentii* CAB 91 in this study does not provide information on the types of PAs involved, future studies must be aimed towards characterising the PAs produced by this yeast strain. This can be achieved by culturing the yeast in liquid media supplemented with the different PAs precursors, *i.e.* arginine, lysine and ornithine, followed by analysing PAs present in the growth media using liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Cloete et al. 2009). In addition, it should be determined which genes encoding for enzymes involved in PAs biosynthesis are present in the genome of *P. laurentii* CAB 91. Molecular techniques, such as amplification of the gene regions using specific primers, as well as cloning and sequencing of these genes, can be used for this purpose. Mutants of *P. laurentii* CAB 91 lacking the genes that encode for the enzymes in PAs biosynthesis can be created using the obtained gene sequences in conjunction with a CRISPR-Cas system to delete the genes from the genome of the yeast strain (Hille and Charpentier 2016). To establish the role that PAs play in the symbiosis between *P. laurentii* CAB 91 and its host plant, these knockout mutants can be tested for their effect on germination, growth and photosynthesis of blue lupin and wheat. For such experiments to be successful, it is imperative to ensure that axenic plants are inoculated with the particular yeast strain. To achieve this, the method described by Kuijken et al. (2015) can be used, since it maintains a sterile rhizosphere

environment, while the rhizosphere can be preferentially inoculated with the desired microorganism.

Papiliotrema laurentii CAB 91 could also have influenced the BNF of blue lupin via two of its other PGP traits, *i.e.* ACC deaminase activity and IAA production. This hypothesis is based on evidence that plant growth promoting rhizobacteria (PGPR) can improve BNF of legumes by producing ACC deaminase and IAA, which increases nodule longevity and ATP levels, respectively (Dashti et al. 1998; Chebotar et al. 2001; Valverde et al. 2006; Figueiredo et al. 2008). Yet, this phenomenon has not been studied for PGPY and, therefore, the influence of ACC deaminase activity and IAA production by *P. laurentii* CAB 91 on blue lupin BNF should be established in future. Little is, however, known about the pathways that PGPY use to produce IAA. Therefore, before the impact of IAA production by *P. laurentii* CAB 91 on ATP levels in blue lupin nodules can be investigated, the pathway used by this yeast strain to produce IAA must be elucidated. This can be accomplished by analysing the intermediates produced by *P. laurentii* CAB 91 in the presence and absence of the precursor tryptophan with high-performance liquid chromatography (HPLC) analysis as described by Nutaratat et al. (2016). Intermediate analysis can indicate whether the yeast strain produces IAA via an indole-3-acetamide, indole-3-acetonitrile, indole-3-pyruvic acid, tryptamine, or tryptophan side chain pathway (Patten and Glick 1996; Nutaratat et al. 2016). In contrast to the lack of knowledge on IAA anabolism in PGPY, it is known that *acdS* encoding for ACC deaminase (Glick 2014; Nascimento et al. 2014) is present in the genome of some yeasts (Minami et al. 1998). Thus, to determine whether nodulation, nodule longevity and BNF efficiency of blue lupin were affected by ACC deaminase activity of *P. laurentii* CAB 91, the molecular techniques mentioned above should also be used to first detect the relevant gene in the genome of this yeast strain. Thereafter, *acdS* mutants of *P. laurentii* CAB 91 can be created and evaluated for their influence on blue lupin nodulation and BNF.

In addition to the potential role of ACC deaminase production by *P. laurentii* CAB 91 in BNF of blue lupin, the activity of this enzyme could have resulted in improved wheat seed vigour. This is owed to the vigour test used in this study being a stress test (Bennett et al. 2004) and ACC deaminase being found to increase seed germination under stress conditions by reducing ‘stress ethylene’ production (Jalili et al. 2009;

Siddikee et al. 2015). To test this hypothesis, *acdS* knockout mutants of *P. laurentii* CAB 91 can be tested for their influence on seed vigour during the cold test. Additionally, seeds treated with these mutants can be evaluated for germination under field conditions to determine if ACC deaminase activity of *P. laurentii* CAB 91 can improve seed vigour in the field.

Considering that gibberellins are known to affect seed germination and some PGPY can produce gibberellic acid (GA_3) (El-Tarabily 2004), it is possible that *P. laurentii* CAB 91 increased blue lupin germination not only through the biosynthesis of PAs, but also via production of GA_3. Therefore, future studies should include *in vitro* analyses for GA_3 production by this yeast strain. This may be achieved by culturing the yeast in complex liquid media (Berrios et al. 2004) whereafter the growth medium can be tested for the presence of GA_3 using LC-MS-MS (Kanno et al. 2016) or ultra performance liquid chromatography–tandem mass spectrometry (Urbanová et al. 2013).

Plant hormone production by PGPM is, however, not limited to GA_3 and IAA, since some PGPR was found to produce cytokinins (CK) (Dobbelaere et al. 2003; Asari et al. 2017) and jasmonic acid (JA) (Abdala et al. 1999; Forchetti et al. 2007). Despite this knowledge, no study to date has been aimed towards determining if PGPY can produce these hormones. Seeing that exogenous application of JA was found to enhance the tolerance of plants to abiotic stress (Qiu et al. 2014) and that CK has several effects on plant growth (Dobbelaere et al. 2003), future studies should be aimed towards establishing whether *P. laurentii* CAB 91 produces these hormones.

By determining all of the above-mentioned facets, the role of *P. laurentii* CAB 91 in the biology of blue lupin and wheat can be established. Considering that the genomes of blue lupin (Hane et al. 2017) and wheat (Zimin et al. 2017) have recently been sequenced, knowledge on the interactions between these plants and *P. laurentii* CAB 91 can be used to develop model systems for identifying genes and their functions involved in plant-microbe interactions. The development of these systems will allow researchers to investigate the effect of complex symbioses on gene expression, since blue lupin and wheat can form such interactions with nodulating bacteria and mycorrhizal fungi, which is currently a limitation of the *Arabidopsis thaliana* system (Busby et al. 2017). These model systems will allow for

the selection of beneficial symbionts for the promotion of crop growth under the threat of climate change, in the end contributing towards the establishment of the 'new green revolution'.

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